DISSECTING THE NEURAL CIRCUITRY

UNDERLYING AVERSION

TO ETHANOL

by

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ABSTRACT

Drugs of abuse, including ethanol, have rewarding and aversive effects, and the balance between them determines drug intake. Although the role of rewarding effects in drug intake has been characterized from a behavioral, neuroanatomical and neurobiological standpoint, the neural mechanisms and neural circuitry underlying aversion to drugs of abuse has not been investigated. The purpose of my dissertation was to dissect the neural circuitry underlying aversion to ethanol. The lateral habenula (LHb) plays an important role in learning driven by aversive outcomes, likely due to the negative modulation of midbrain monoaminergic systems. However, the role of the LHb in regulating ethanol intake is not known. To begin characterizing the role of the LHb in ethanol-directed behaviors, I studied voluntary ethanol consumption, operant self-administration, yohimbine-induced reinstatement of ethanol-seeking, and conditioned taste aversion (CTA) in sham- and LHb-lesioned rats. I found that lesions of the LHb increase the rate of escalation of ethanol intake, increase operant ethanol-seeking, block yohimbine-induced reinstatement of ethanol-seeking, and attenuate ethanol-induced conditioned aversion. My results suggest that the LHb contributes to multiple facets of ethanol-directed behaviors, likely by mediating learning driven by the aversive effects of ethanol. I further investigated the afferents and efferents of the LHb that may be critical for ethanol-associated

behaviors. I found that the lesions of the rostromedial tegmental nucleus (RMTg), a major efferent target of the LHb, increased voluntary ethanol consumption and accelerated extinction of ethanol-induced CTA. Thus, the LHb and RMTg play complementary roles in regulation of voluntary ethanol intake, most likely by mediating persistence of ethanol-induced aversive effects. With regard to the afferents of the LHb, my results show that the projection from the lateral hypothalamus (LH) to the LHb is critical for regulation of voluntary ethanol intake. Thus, I propose that the LH-LHb-RMTg circuit may be critical in regulation of ethanol intake.

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LIST OF ABBREVIATIONS

+3, 3’-diaminobenzine; DAB

α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AMPA

Analysis of Variance; ANOVA

Di-N-Butyl phthalate in xylene; DPX

Excitatory post-synaptic current; EPSC

Fixed-ratio, FR

Gamma-aminobutyric acid; GABA

Intramuscular; i.m.

Intraperitoneal; IP

Intravenous; i.v.

N-methyl-D-aspartate; NMDA

Post-synaptic density; PSD

Standard deviation; S.D.

Standard error of mean; S.E.M

CHAPTER 1

INTRODUCTION

The habenula is an epithalamic structure that is phylogenetically well conserved across species, suggesting that it regulates processes crucial for survival (Aizawa et al., 2011; Stephenson-Jones et al., 2012). It is divided into the medial habenula (MHb) and the lateral habenula (LHb), which are anatomically and physiologically distinct from one another (Bianco and Wilson, 2009; Hikosaka, 2010). Over the past decade, there has been a growing interest in the LHb. This interest has arisen due to its strategic location, which allows LHb to integrate information from the limbic system and the basal ganglia through the stria medullaris (Herkenham and Nauta, 1977) and send efferent projections to midbrain dopaminergic (DA) systems, such as the ventral tegmental area (VTA) and substantia nigra pars compacta (SNc) and serotoninergic (5-HT) systems, such as the dorsal raphe nucleus (DRN) and the median raphe nucleus (MnR) (Herkenham and Nauta, 1979). Thus, the LHb links the forebrain to the midbrain monaominergic systems. Given the role of the monoaminergic systems in regulating cognition, mood (Nestler and Carlezon, 2006), motivated behavior (Wise, 2004), and reward-related learning (Schultz, 2007b), and the fact that LHb

modulates these systems, it is not surprising that the LHb has been implicated in a range of neuropsychiatric conditions, including depression (Lecca et al., 2014), schizophrenia, and drug addiction (Lecca et al., 2014). This review will summarize the role of the LHb in regulating reward-related learning, cognition, memory and stress-induced behavioral responses, which could potentially contribute to the involvement of the LHb in drug addiction. The review then aims to demonstrate an emerging role for the LHb in behaviors associated with different drugs of abuse and will attempt to identify the possible afferent and efferent connections that may be instrumental in mediating effects of LHb on drug-directed behaviors.

The LHb: afferents and efferents

The LHb receives input from the limbic forebrain, basal ganglia and cortical structures through the stria medullaris (Herkenham and Nauta, 1977). The limbic afferent projections preferentially target the medial subdivision of the LHb, and basal ganglia structures preferentially innervate the lateral subdivision of the LHb (Herkenham and Nauta, 1977). In this regard, the medial subdivision of the LHb receives inputs primarily from the lateral hypothalamus (LH), lateral preoptic area (LPO), and the anterior insular (AI), anterior cingulate (ACC), prelimbic and infralimbic cortices (Kowski et al., 2008; Li et al., 2011; Kim and Lee, 2012; Poller et al., 2013). In contrast, the lateral subdivision of the LHb receives a major projection from the entopeduncular nucleus (EPN) (rodent homologue of primate globus pallidus internal [GPi]) (Parent et al., 2001; Shabel

et al., 2012). The differential projections to the medial and lateral subdivisions of the LHb suggest that the regions may serve different physiological functions; however, studies testing this hypothesis are scarce. The potential challenge in dissecting out the functions of the medial and lateral subdivisions of LHb is the difficulty in targeting these subdivisions without encroaching on to the neighboring subdivision.

Other projections to the LHb include the ventral pallidum (VP), diagonal band of Broca (DBB), bed nucleus of the stria terminalis (BNST), ventral tegmental area (VTA), and median raphe (MnR) (Herkenham and Nauta, 1977; Dong and Swanson, 2006; Stamatakis et al., 2013; Root et al., 2014). Importantly, the LH, LPOA, EPN and VP are the output structures of the extended amygdala, lateral septal-preoptic system, the dorsal striatopallidal system, and the ventral striatopallidal system, respectively. Thus, the LHb integrates information from these major macrosystems in the brain (Geisler and Trimble, 2008) and is critically positioned to receive direct or indirect information from a number of structures related to reward (VTA and VP), motor output (EPN/GPi), cognition and affect (prefrontal cortex), and stress and anxiety (LH, BNST) (Figure 1.1).

Information funnels out of the LHb through the fasciculus retroflexus (FR) (Herkenham and Nauta, 1979). The efferent projections terminate in midbrain DAergic (VTA, SNc), 5-HTergic (DRN and MnR), and acetylcholine (Ach)-rich (pedunculopontine and laterodorsal tegmental nuclei) nuclei (Herkenham and Nauta, 1979). The LHb is known to exert an inhibitory influence on DA neurons in

the VTA and SNc (Christoph et al., 1986) and 5-HT neurons in the DRN and MnR (Wang and Aghajanian, 1977). Accordingly, acute inactivation of the LHb increases DA turnover in the nucleus accumbens and prefrontal cortex, which are the main efferent targets of DA neurons in the VTA (Lisoprawski et al., 1980; Lecourtier et al., 2008). Further, LHb stimulation reduces 5-HT release in the raphe and caudate of cats (Reisine et al., 1982); this effect is blocked by lesions of the FR (Kalen et al., 1989). At odds with this inhibitory influence is the fact that most LHb neurons are glutamatergic. Thus, it was postulated that inhibition of the midbrain monoaminergic systems is mediated by a disynaptic pathway. A recently identified brain structure called the rostromedial tegmental nucleus (RMTg; also called the tail of the ventral tegmental area [tVTA]), which is a GABAergic nucleus, receives afferent projections from the LHb and projects to the VTA, SNc, DRN, and MnR, and thus serves as the relay structure (Jhou et al., 2009b; Kaufling et al., 2010a; Balcita-Pedicino et al., 2011; Hong et al., 2011; Sego et al., 2014). Furthermore, similar to LHb stimulation, RMTg stimulation inhibits spontaneous firing of midbrain DA neurons (Lecca et al., 2012). That said, it should be noted that the LHb also sends fewer, yet some, direct projections to VTA, DRN, and MnR (Goncalves et al., 2012; Sego et al., 2014). Collectively, these data suggest that the LHb exerts a ‘brake pedal’ on the midbrain DA and 5-HT systems through a disynaptic pathway involving the RMTg.

Role of the LHb in reward and aversion

Predicting reward and punishment and changing behavior by assessing the difference between expected and actual outcomes is critical for survival in all species (Schultz et al., 1997). When a reward is predicted, a behavioral response is based on the most recent history of reward, which is called the reward – prediction error (RPE) (Schultz, 1998). VTA DA neurons are thought to encode RPE (Schultz, 2007b). That is, if the reward earned by a behavioral response is greater than predicted or when an unpredicted reward is delivered, the DA neurons phasically increase firing, resulting in a positive prediction error (PPE). Conversely, if the reward achieved by a behavioral response is smaller than predicted or a predicted reward is not delivered, the DA neuron firing is suppressed, resulting in a negative prediction error (NPE) (Schultz, 2007b). This change in firing of DA neurons serves as a learning signal: during PPE, the phasic DA neuron firing elevates DA levels in terminal fields, which serves as a GO signal, or consolidates the performance of the behavior that led to the reward (Kravitz et al., 2010). During NPE, the suppression of DA neuron firing results in decreased DA release in terminal fields, which serves as a NO-GO signal or decreases the likelihood that the behavior associated with the lesser outcome will be executed the next time (Nakamura and Hikosaka, 2006).

The LHb exhibits phasic changes in activity in association with RPEs. Specifically, LHb neuron firing is inhibited when unpredicted reward is delivered (i.e., PPE) and excited when a predicted reward is not delivered (i.e., NPE) (Matsumoto and Hikosaka, 2007). In addition, habenular activity correlates with

NPE in humans (Salas et al., 2010). Given that the LHb sends excitatory afferents to the RMTg, the activity of RMTg neurons is similar to that of LHb neurons: RMTg neuron activity is inhibited in the case of PPE and excited in the case of NPE (Jhou et al., 2009a; Hong et al., 2011). This pattern of firing in LHb and RMTg is opposite to that seen in putative midbrain DA neurons which are excited by PPE and inhibited by NPE (Fiorillo et al., 2003). Finally, lesions of the habenula weaken PPE responses and make them less reliable and completely abolish NPE responses in VTA DA neurons (Tian and Uchida, 2015). Together, these data suggest that the LHb may play a role in encoding RPEs and may propagate that encoding to VTA DA neurons.

LHb neurons also show phasic excitation in response to aversive events such as punishment or sensory cues predicting punishment, implying that the LHb may encode aversion (Matsumoto and Hikosaka, 2009a). This hypothesis is supported by results of other studies showing that various aversive events, such as food deprivation, restraint stress, and footshock, cause *c*-fos (marker for neuronal activity) induction in the medial subdivision of the LHb (Wirtshafter et al., 1994; Timofeeva and Richard, 2001; Brown and Shepard, 2013). As in the case of encoding RPEs, increased firing of LHb neurons in response to an aversive stimulus presumably is then relayed to downstream targets, such as the VTA DA neurons, that respond to aversive stimuli with either phasic excitation or inhibition (Matsumoto and Hikosaka, 2009b). In fact, a direct projection from the LHb to VTA supports conditioned place aversion via DAergic projections to the mPFC (Lammel et al., 2012). Further, studies in humans reveal strong functional

connectivity between the habenula and the VTA in response to aversive stimuli (Hennigan et al., 2015). Finally, both conditioned and unconditioned aversive events also cause *c-fos* induction in RMTg neurons, which project to VTA DA neurons and receive afferents from LHb (Brown and Shepard, 2013), providing a circuit level basis for LHb activity to drive DA neuron inhibition by aversive events. Collectively, this evidence suggests that aversive information can be transmitted from LHb to midbrain DA neurons via two distinct synaptic connections: - 1) direct connection from LHb to VTA DA neurons; 2) indirect connection from LHb to VTA DA neurons through RMTg.

Afferent connections that may convey reward and aversion-related information to LHb

A number of brain regions may provide the afferent drive to the LHb neurons necessary for them to encode RPEs and aversive events. First, the VTA appears to send both inhibitory and excitatory afferents to the LHb. On the one hand, a recent study suggests that a GABAergic afferent projection from VTA neurons to the LHb promotes reward-related behavior (Stamatakis et al., 2013). This study used an optogenetic approach combined with electrophysiology, genetically-targeted neuronal tracing, and behavioral analyses to show that optogenetic stimulation of terminals of a unique population of VTA neurons in the LHb inhibited LHb and RMTg via GABA neurotransmission. This stimulation increased the spontaneous activity of VTA neurons and resulted in preference for the side of the chamber paired with the optogenetic stimulation. In addition, mice

in that study would nose-poke to receive the optogenetic stimulation, suggesting that the stimulation of this VTA-LHb afferent pathway is positively reinforcing (Stamatakis et al., 2013). It is thus plausible that activation of this GABAergic mesohabenular pathway in response to rewarding stimuli inhibits LHb neuronal activity, which then disinhibits VTA DA neuronal activity, thereby promoting approach behaviors.

On the other hand, another recent study has identified a VTA afferent to the LHb that appears to promote encoding of aversive events via glutamate signaling in the LHb. Light activation of the input from this VGlut2-positve VTA neurons to the LHb promotes aversive conditioning (Root et al., 2014). The opposing effects of activation of different afferent projections from VTA to the LHb suggest that rewarding and aversive stimuli may activate divergent subpopulations of VTA neurons, thereby producing projection-specific effects in LHb activity and, thus, contrasting behaviors.

A second afferent projection to the LHb that may convey reward and aversion-related information is the EPN. Optogenetic stimulation of the EPN terminals in the LHb causes real-time place aversion of the chamber in which the stimulation was received, suggesting that the activation of the EPN to LHb projection is aversive (Shabel et al., 2012). In support of this idea, most of the GPi (primate homolog of EPN) neurons projecting to the LHb encode NPEs, as do LHb neurons, and the NPE-related activity in the GPi precedes that observed in the LHb neurons (Hong and Hikosaka, 2008). These findings suggest that the GPi/EPN afferent to the LHb may convey negative motivational state information

to the LHb.

A final afferent projection to the LHb that may convey reward value-related signals is the VP. Both VP and LHb neurons encode reward but in opposite directions (Hong and Hikosaka, 2013). Taken together, these findings suggest that LHb is critically positioned to receive reward- and aversion-related information from a variety of inputs, including but not limited to the VTA, EPN/GPi, and VP.

LHb facilitates learning driven by RPEs

DA neurons facilitate reward-driven as well as punishment-driven learning by signaling RPEs (Bromberg-Martin et al., 2010). Given the evidence, reviewed above, that such RPEs may be driven by LHb activity, it is possible that LHb activity contributes to learning by affecting DA neuron activity and, hence, guiding behavior. In fact, when macaques perform a visually guided saccade task, postsaccadic LHb stimulation increases saccade latency during subsequent trials, suggesting that LHb may be involved in learning to suppress actions which lead to less than optimal outcomes (Matsumoto and Hikosaka, 2011). Similarly, LHb stimulation paired with a correct avoidance response during the acquisition phase worsens avoidance learning, underscoring the importance of normal functioning of LHb in learning driven by aversive outcomes (Shumake et al., 2010). Furthermore, optogenetic stimulation of LHb terminals in the RMTg leads to conditioned, active, and passive avoidance (Stamatakis and Stuber, 2012). In fact, mice will nose-poke in order to avoid stimulation of the LHb terminals in the

RMTg, suggesting that stimulation of the LHb-RMTg pathway is negatively reinforcing (Stamatakis and Stuber, 2012). Finally, in zebrafish, the LHb-MnR pathway is indispensable for active avoidance learning, suggesting that LHb modulation of downstream 5-HT neurons may also contribute to this form of learning (Amo et al., 2014). Together, this evidence suggests a central role for the LHb in facilitating learning in response to salient stimuli, including stimuli that signal worse than predicted outcomes via modulation of midbrain monoaminergic systems.

Role of LHb in cognition, learning and memory

The LHb plays a broad role in spatial learning and memory, implicating it in cognitive processes (Lecourtier and Kelly, 2007). Aged rats showing impairments on the Morris water-maze test have lower metabolic activity in the LHb as compared to young rats, suggesting that the LHb may be involved in spatial learning (Villarreal et al., 2002). This finding is consistent with results demonstrating that lesions of the habenula cause deficits on the Morris-water maze test (Lecourtier et al., 2004) and inactivation of the LHb produces deficits in spatial configuration processing (Goutagny et al., 2013). One of the plausible downstream mechanisms for LHb effects on these cognitive processes is that lesions of the habenula increase firing of 5-HT neurons (Wang and Aghajanian, 1977), thereby increasing 5-HT levels in various brain areas, including the hippocampus. The induction of long-term depression (LTD) in the hippocampus is prevented by 5-HT, which could thus contribute to the cognitive deficits

(Lecourtier et al., 2004). Also, lesions of the habenula impair synaptic plasticity in the fimbria-accumbens pathway (Lecourtier et al., 2006), which could account for some of the cognitive deficits. Together, this evidence indicates that the LHb is crucial in cognitive processing, most likely by modulating 5-HT systems in the midbrain.

The habenula has also been postulated to play a role in impulsivity and attention. Bilateral lesions of the habenula increase premature responding and produce progressive alterations in accuracy in a 5-choice serial reaction time task (5-CSRTT) (Lecourtier and Kelly, 2005). The increase in premature responding could be considered an increase in impulsivity, whereas alterations in accuracy are thought to reflect an attentional deficit. Further, inactivation of the LHb increases DA in terminal fields, including the NAcc and mPFC (Lecourtier et al., 2008), which may be responsible for the deficits in performance in the 5-CSRTT. With regard to memory, LHb inactivation during acquisition prevents long-term stability of an aversive memory while leaving the formation of the memory intact, suggesting a complex role of the LHb in storage of long-term memories (Tomaiuolo et al., 2014). The deficit produced by LHb inactivation is reversed by DA manipulations in the mPFC, indicating that LHb may be involved in long-term persistence of memories by modulation of DA (Tomaiuolo et al., 2014).

Afferent connections that may convey a cognition and memory-related input to the LHb

A potential source of input to the LHb that may convey cognition-related information is the prelimbic cortex (Kim and Lee, 2012), which has been implicated in higher-order processing related to memory, attention, and coordinating goal-oriented behaviors (Dalley et al., 2004; Vertes, 2006). This suggests that input from the prelimbic as well as other cortical inputs, including those from the ACC, which is critical for choice accuracy (Chudasama et al., 2003), can relay cognitive information to the LHb, given that these areas project to the LHb (Vadovicova, 2014). In fact, one recent report implicated glutamatergic transmission to the LHb in encoding and retrieval of spatial learning (Mathis et al., 2015). The prefrontal cortex could be this source of excitatory input to the LHb (Kim and Lee, 2012). In addition, ACC and medial prefrontal cortex (mPFC) are necessary for expression and storage of long-term memories (Ding et al., 2008; Gonzalez et al., 2014). This finding, coupled with evidence of a strong projection from the ACC and mPFC to the LHb, implicates these projections in long-term storage of memories (Kim and Lee, 2012; Vadovicova, 2014). In summary, these findings suggest that cortical inputs to the LHb (prelimbic, ACC, mPFC) may convey cognitive information related to spatial learning, attention, and long-term storage of memories.

Role of LHb in stress-induced behavioral responses

The LHb seems to play a crucial role in stress-induced behavioral and neurochemical responses. For instance, lesion of the habenula reduces prepulse inhibition (PPI) after stress; however, the lesion has no effect on PPI in the absence of stress (Heldt and Ressler, 2006). Further, the deficit in PPI in lesioned animals subjected to conditioned fear is blocked by clozapine, which is a broad-spectrum antagonist of DA, 5-HT and other receptors, suggesting that the habenula is involved in stress-dependent regulation of monoaminergic systems (Heldt and Ressler, 2006). Also, lesions of the habenula decrease avoidance learning when it is tested under high-stress conditions (Thornton and Bradbury, 1989). Habenular lesions also block development of learned helplessness in response to inescapable shock, which has been attributed to a lack of increase in 5-HT levels in the DRN of rats with lesions of the habenula, suggesting that this nucleus is essential for development of a depressive phenotype after an uncontrollable stressor (Amat et al., 2001). Finally, inactivation of the LHb reduces anxiety-related behavior and stress-induced reinstatement of ethanol- and cocaine-seeking in response to a pharmacological stressor such as yohimbine (Gill et al., 2013; Haack et al., 2014). Together, these results suggest a unique modulatory role for the LHb on a diverse range of stress-related behaviors.

Afferents that may convey a stress and anxiety-related input to the LHb

A number of brain regions may provide a stress- and anxiety-related input to the LHb. Parts of the extended amygdala, including the BNST, project to the LHb (Herkenham and Nauta, 1977; Dong and Swanson, 2006). Yohimbine increases norepinephrine and cortcicotropin-releasing hormone (CRH) in the amygdala and the BNST. In addition, the LHb receives a major projection from the LH (Poller et al., 2013), which controls glucocorticoid release and initiates the activation of the hypothalamic-pituitary adrenal axis (HPA), thereby regulating the stress response (Johnston et al., 1988). Thus, it seems plausible that LHb receives stress-related information from structures such as the LH and BNST, and conveys that stress-related information to the midbrain.

How does LHb contribute to drug intake?

Several studies have reported an integral role for the LHb in drug-directed behaviors, suggesting involvement of the LHb in the process of drug addiction (Friedman et al., 2010; Jhou et al., 2013; Haack et al., 2014) (Table 1.1). A potential mechanism by which the LHb may mediate drug intake is by regulating the rewarding and aversive aspects of drug intake. Drugs of abuse have both rewarding and aversive properties, and the relative balance between the two governs drug intake (Riley, 2011; Verendeev and Riley, 2013). A meta-analysis concluded that there exists an inverse correlation between the aversion experienced to alcohol and voluntary alcohol consumption, suggesting that aversive effects of alcohol may limit alcohol intake (Green and Grahame, 2008).

Similar results have been obtained with cocaine (Ettenberg et al., 2015). In humans, low aversive responses to alcohol are predictive of higher risk for alcohol-use disorders and binge alcohol intake (King et al., 2011). Since the LHb is critical in the processing of both reward and aversion, it could be a likely point in the neural circuitry where these opposite properties merge. Hence, the LHb may potentially control both appetitive and aversive aspects of drug intake. I provide evidence in Chapter 2 to suggest that the LHb regulates ethanol consumption by mediating ethanol-induced conditioned aversion. Similar results also are reported in the dissertation regarding the role of the RMTg (Chapter 4) in the behavioral response to ethanol.

It is also possible that LHb regulates drug intake by altering impulsive behavior and cognition, as reviewed above. Addictive behaviors are associated with significantly elevated impulsive choice (MacKillop et al., 2011). However, it is not clear whether impulsivity is a cause or consequence of drug abuse. There is evidence that impulsivity could make individuals susceptible to initiating drug use. For example, elevated impulsive action has been observed in first-degree relatives of substance abusers, who themselves had no history of drug abuse (Ersche et al., 2012a; Ersche et al., 2012b). Further, elevated impulsive choice during development was associated with subsequent initiation of smoking (Audrain-McGovern et al., 2009) and even drug use 20 years later (Ayduk et al., 2000). Since lesions of the habenula cause impulsivity in rats (Lecourtier and Kelly, 2005), it follows that habenular activity may contribute to drug-seeking by regulating impulsive choice and action.

Finally, LHb can contribute to drug intake due to its role in stress-induced behavioral adaptations. Stress is known to escalate drug intake and is also a critical trigger in relapse even after long periods of abstinence (Breese et al., 2005). Given the role of the LHb in mediating behavioral adaptations in response to stress (as outlined above), the LHb may be critically engaged during stress-induced increases in drug consumption and stress-induced relapse. I show in Chapter 2 that lesions of the LHb block stress-induced reinstatement of ethanol-seeking. However, I have also found that lesions of the RMTg have no effect on stress-induced reinstatement of ethanol-seeking (Chapter 4), suggesting an alternate efferent pathway from the LHb for mediating stress-induced increase in ethanol-seeking.

Thus, the LHb could be involved in drug addiction due to its role in reward and aversive processing, cognitive processing and stress-induced behavioral responses. The following paragraphs summarize the results of studies investigating the role of the LHb and its major efferent target, the RMTg, in behaviors associated with specific drugs.

Effects of cocaine on the LHb and RMTg

Cocaine alters the neuronal activity in the LHb-RMTg pathway. Acute and chronic cocaine causes *c-fos* induction in the LHb (Zahm et al., 2010) and the RMTg (Geisler et al., 2008; Kaufling et al., 2010b). Specifically, cocaine injections or cocaine self-administration causes *c-fos* expression in RMTg neurons projecting to VTA neurons (Kaufling et al., 2010a), suggesting a

potential downstream mechanism mediated by DA. Further, in brain slices, cocaine increases LHb neuronal firing, and this effect is abolished by AMPA and NMDA receptor blockade, implying that cocaine increases LHb neuronal firing by enhancing glutamatergic transmission (Zuo et al., 2013). Likewise, a recent report showed that cocaine increases AMPA receptor-mediated EPSCs in LHb neurons projecting to the RMTg (Maroteaux and Mameli, 2012). Together, these data show that cocaine activates the LHb.

Whether cocaine-induced activation of LHb is modulated by DA is subject to debate. The principal mechanism of action of cocaine is inhibition of the DA transporter (DAT), which increases DA levels in the synapse. The LHb expresses DAT as well as DA receptors (D1R and D2R) (Dubois et al., 1986; Savasta et al., 1986). It has been proposed that cocaine increases extracellular DA in the LHb, which then binds to the D1R and D2R presynaptically to increase glutamate release from the glutamatergic terminals synapsing onto the LHb neurons, thereby activating the LHb (Good et al., 2013). However, in a later study, the same group suggested that cocaine and other DAT inhibitors influence LHb activity via norepinephrine (NE), not DA, actions on D4 DA receptors (Root et al., 2015). Regardless of the neurotransmitter involved, cocaine increases LHb neuron firing, thereby increasing the excitatory drive onto RMTg neurons which then inhibit VTA DA neurons. In addition, cocaine administration causes long-lasting decreases in GABA terminal immunolabeling in the LHb, indicating that cocaine reduces presynaptic inhibitory activity, thus causing disinhibition of LHb neurons (Meshul et al., 1998). Together, the data show that cocaine has a net

facilitatory effect on LHb neuron activity.

Jhou and colleagues (2013) demonstrated that the LHb-RMTg pathway is critical for mediating learned avoidance responses to cocaine (Jhou et al., 2013). In contrast to the studies above, cocaine caused an initial inhibition of LHb neurons; however, a subset of these neurons then showed rebound excitation (Jhou et al., 2013). The time course of the biphasic activity of LHb neurons coincides with the behavioral effects of cocaine in a place-conditioning paradigm- that is, initial reward followed by aversion, suggesting that the initial inhibition of LHb neurons by cocaine manifests as reward, whereas the later rebound excitation manifests as aversion. Interestingly, *c-fos* immunohistochemistry revealed that the LHb neurons that show rebound excitation in response to cocaine preferentially project to the RMTg. Thus, the later rebound excitation of LHb neurons by cocaine increases RMTg neuronal activity, which then inhibits VTA DA neurons. The electrophysiological evidence is corroborated by functional evidence showing that RMTg and FR lesions eliminate the avoidance response to cocaine on a runway-alley task, suggesting that increase in the LHb-RMTg pathway activity is critical for mediating the cocaine-induced aversive motivational state (Jhou et al., 2013). Interestingly, this effect of RMTg on avoidance is time-dependent, as optogenetic inhibition of the RMTg 15-20 mins, but not 0-10 mins, after the infusion of cocaine abolishes cocaine-induced avoidance (Jhou et al., 2013). In agreement, a different study has shown that long-lasting adaptations seen in the LHb following cocaine administration are critical for the emergence of cocaine-induced negative symptoms (Meye et al.,

2015). Together, these data suggest that the LHb-RMTg pathway is crucial for mediating a cocaine-induced aversive state, likely by its actions on downstream VTA DA neurons.

The above outlined evidence indicates that the LHb could be involved in mediating aversive aspects of cocaine intake and perhaps can therefore control cocaine intake. Indeed, deep-brain stimulation (DBS) of the LHb attenuates cocaine self-administration and, cue- and cocaine-induced reinstatement, and accelerates extinction of cocaine-seeking (Friedman et al., 2010). On the other hand, the same study showed that chemical lesions of the LHb prevent the rats from attaining the extinction criterion. Further, activation of the RMTg consolidates extinction memory in a cocaine-seeking paradigm (Huff and LaLumiere, 2014), suggesting that the LHb may mediate its effects on cocaine-seeking via RMTg. Also, cocaine self-administration increases expression of NR1, GluR1, and PSD95 in the VTA, all of which enhance synaptic plasticity, which underlies maladaptations in reward and motivational circuits, causing further drug intake (Friedman et al., 2010). Interestingly, LHb stimulation normalizes the levels of NR1, GluR1, and PSD95 in VTA, which could underlie the reduced cocaine-seeking in LHb-stimulated rats (Friedman et al., 2010). Thus, LHb could be a potential candidate for DBS to reduce cocaine intake in addicts.

It is important to note that the efficacy of DBS of the LHb in reducing cocaine-seeking depends on the history of cocaine use. Continuous cocaine causes degeneration in the FR, which has been hypothesized to lead to drug-

induced psychosis since the midbrain DA neurons are no longer under the inhibitory control of the LHb (Ellison, 2002; Lax et al., 2013). Consistent with this finding, stimulation of the LHb had no effect on cocaine-seeking when rats administered high doses of cocaine at which FR degeneration was observed, as opposed to reduced cocaine-seeking when self-administering low and intermediate doses of cocaine at which no FR degeneration was observed (Lax et al., 2013). This finding could have important clinical implications for DBS of the LHb in reducing cocaine-seeking. Whether or not DBS will have a beneficial effect on cocaine-seeking can be predicted by the integrity of the FR by diffusion tensor imaging (DTI) in a clinical setting (Yadid et al., 2013).

The LHb has also been implicated in mediating relapse to cocaine after a period of abstinence. The main impediment in treating drug addiction, including cocaine addiction, is the chronically relapsing nature of this disease. Individuals who have maintained abstinence for a long duration of time also show high rates of relapse (Hunt and Bespalec, 1974; Miller, 1991). There are several triggers that can cause an abstinent individual to relapse: cues and context related to the drug, the drug itself, and stress (Larimer et al., 1999). LHb neurons show altered metabolic activity and *c-fos* expression in response to cocaine-paired cues, suggesting that the LHb could be involved in cue-induced relapse to cocaine (Brown et al., 1992; Franklin and Druhan, 2000; Knapp et al., 2002). Further, inactivation of the LHb prevents stress-induced reinstatement of cocaine-seeking (Gill et al., 2013). Also, mice that are more vulnerable to cocaine-primed reinstatement have higher levels of c-*fos* in the LHb (Brown et al., 2010). Thus,

the LHb seems to play a broad role in mediating relapse to cocaine-seeking, irrespective of the trigger.

Effects of ethanol on the LHb and RMTg

Local cerebral glucose utilization (LCGU) and *c-fos* studies have shown that LHb neurons are sensitive to ethanol administration (Williams-Hemby and Porrino, 1994; Strother et al., 2005; Zuo et al., 2013). Specifically, in rat brain slices, ethanol increases LHb neuronal firing by elevating presynaptic glutamate release, partly acting through D1 receptors (Zuo et al., 2013). In addition, ethanol causes a dose-dependent increase in RMTg neuronal firing (Melis et al., 2014).

A recent study from our laboratory investigated the role of the LHb in ethanol-associated behaviors (Chapter 2). Therein, I show that bilateral electrolytic lesions of the LHb increase voluntary alcohol consumption and operant responding, while blocking yohimbine-induced reinstatement of ethanol-seeking (Haack et al., 2014). The increase in voluntary intake and operant responding seen in LHb-lesioned rats may be due to deficits in aversive learning, given that LHb-lesioned rats recover faster from ethanol-induced aversion in a CTA paradigm (Haack et al., 2014). The role of the LHb in ethanol-induced conditioned aversion is further solidified by a recent report that found that inhibiting LHb activity abolishes conditioned-place aversion (CPA) to ethanol (Zuo et al., 2013). However, I show in Chapter 3 of the present dissertation that the LHb plays little role in mediating acute aversive effects of ethanol, including motor impairment, sedation, and ethanol-withdrawal-induced anxiety.

In this dissertation, I also show that RMTg-lesioned rats voluntarily consume more ethanol than sham-lesioned rats and also show accelerated extinction of ethanol-induced CTA (Chapter 4), suggesting that the RMTg, like the LHb, is crucial for controlling ethanol consumption, most likely by attenuating the persistence of ethanol-induced aversive learning. Further support for this conclusion comes from a recent study that reported no effect of ethanol on RMTg neuronal firing in a rat strain bred for high ethanol preference (sP rats) in comparison to an ethanol-induced increase in RMTg neuronal firing in a rat strain selectively bred for ethanol aversion (sNP rats) (Melis et al., 2014). The above evidence thus suggests that an ethanol-induced increase in RMTg neuronal activity may serve as an aversive signal that reduces subsequent ethanol intake.

In addition, I also show in Chapter 5 of the present dissertation that afferents to the LHb are critical for controlling ethanol consumption, as rats with lesions of the SM drink more ethanol than controls in an intermittent ethanol access (IEA) paradigm. To further investigate the inputs to the LHb that are crucial in regulation of ethanol consumption, I cross-lesioned the LH and the LHb, as well as the VP and the LHb, in separate experiments. I found that input from the LH to the LHb regulates ethanol consumption, whereas the projection from VP is not critical for this behavior. Thus, I propose that the LH-LHb-RMTg circuit is critical for regulation of voluntary ethanol consumption.

Effects of opioids on the LHb and RMTg

The effect of opioids on LHb firing and the exact role of the LHb in opioid addiction have not been investigated. In contrast, several studies have probed the effect of opioids on RMTg firing, given that this nucleus has one of the highest expressions of μ-opioid receptors in the brain (Jhou et al., 2009b). Systemic administration of morphine inhibits the spontaneous firing rate of putative RMTg neurons, a μ-opioid receptor antagonist blocks this effect (Lecca et al., 2011). In addition, morphine reduces the duration of RMTg-induced inhibition of DA neurons, thus disinhibiting them (Lecca et al., 2012). Also, pharmacological inactivation of the RMTg blocks VTA DA neuron activation produced by i.v. morphine (Jalabert et al., 2011). Together, this evidence suggests that the RMTg is an important site for regulating opioid action on VTA DA neurons. Further, during protracted opiate withdrawal, the regulation of VTA DA neurons by the RMTg is altered, such that the RMTg can no longer disinhibit VTA DA neurons (Kaufling and Aston-Jones, 2015). Thus, the RMTg may play a vital role in acute opioid actions, as well as during protracted withdrawal.

The LHb may be crucial in mediating relapse to opioids. For example, the LHb shows *c-fos* induction during cue-induced reinstatement of heroin-seeking (Zhang et al., 2005), and mice that are more vulnerable to relapse to morphine have elevated LHb activity (Madsen et al., 2012). Future studies should investigate the effect of opioids on LHb neuronal activity and how the LHb and RMTg modulate different aspects of opioid addiction.

Effects of nicotine on the LHb and RMTg

Data supporting the involvement of the LHb in nicotine intake are scarce. However, a plethora of literature exists suggesting that the MHb plays a role in nicotine intake, aversion and addiction (Baldwin et al., 2011; Frahm et al., 2011; Fowler and Kenny, 2014; Velasquez et al., 2014). However, full discussion of the involvement of the MHb in nicotine addiction is beyond the scope of this dissertation.

With regard to the involvement of the LHb and the RMTg in nicotine-associated behaviors, acute and chronic administration of nicotine increases *c-fos* expression in the LHb (Mathieu-Kia et al., 1998); however, the effects of nicotine on LHb neuronal firing remain to be determined. Nicotine robustly stimulates the firing of putative RMTg neurons, an effect that is blocked by a nicotinic acetylcholine receptor antagonist (nAChR) (Lecca et al., 2011). The ability of nicotine to stimulate RMTg neuron firing is attributed to enhancement of glutamate release from habenular excitatory afferents containing alpha-7-containing nAChRs on presynaptic terminals (Lecca et al., 2011). LHb lesions disrupt nicotine-induced anxiety, suggesting that the LHb could be playing a potential role in mediating aversive effects of nicotine (Casarrubea et al., 2015). The functional consequences of nicotine-induced potentiation of LHb (*c-fos*) and RMTg (electrophysiology) activity on nicotine intake remain to be investigated.

Conclusions

Together, results above suggest that the LHb plays a critical role in intake of most drugs of abuse most likely by mediating drug-induced aversive learning (Jhou et al., 2013; Zuo et al., 2013; Haack et al., 2014). Given that the LH, LHb, and RMTg control ethanol intake, they present novel targets that can be manipulated pharmacologically to reduce ethanol intake in vulnerable populations. Future studies should address synaptic and cellular changes that occur in the LHb and its downstream targets (e.g., the RMTg) after prolonged exposure to drugs of abuse (as opposed to acute effects), which would more accurately mimic human addiction. In conclusion, there is emerging data implicating the LHb and its major efferent target, the RMTg, in drug-associated behaviors.

Goal of dissertation

The primary goal of this dissertation was to dissect the neural circuitry that underlies aversion to ethanol and hence can potentially regulate ethanol consumption. Identifying anatomical regions and their role in ethanol-directed behaviors will enhance our understanding of how aversion to ethanol can contribute to ethanol intake and assist in the development of possible therapeutics.

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Figure 1.1 Afferent and efferent connectivity of LHb. LHb receives input from VTA, VP, EPN, LH, BNST, and PFC. Afferents which relay reward-related information are shown in yellow, aversive-related information in blue, stress- and anxiety-related information in orange, and cognition-related information in brown. Green arrow indicates excitatory projection, red indicates inhibitory projection, and gray dashed line indicates unknown nature of projection. The thickness of the arrows indicates the strength of the projection. There is evidence for both an excitatory and inhibitory projection from VTA to LHb, depending upon the nature of the VTA neurons from which the projection arises (Stamatakis et al., 2013; Root et al., 2014). It is important to note that none of the afferent inputs are exclusive in one physiologic function, for example, although LH is shown as an afferent input which conveys stress and anxiety related information to LHb, it is also possible that it plays a role in relaying reward-related information to LHb. LHb integrates information from these afferent inputs and bi-directionally modulates midbrain DA and 5-HT systems via direct or indirect projections involving the RMTg. It is important to note that this is not an exhaustive list of afferents and efferents of the LHb.

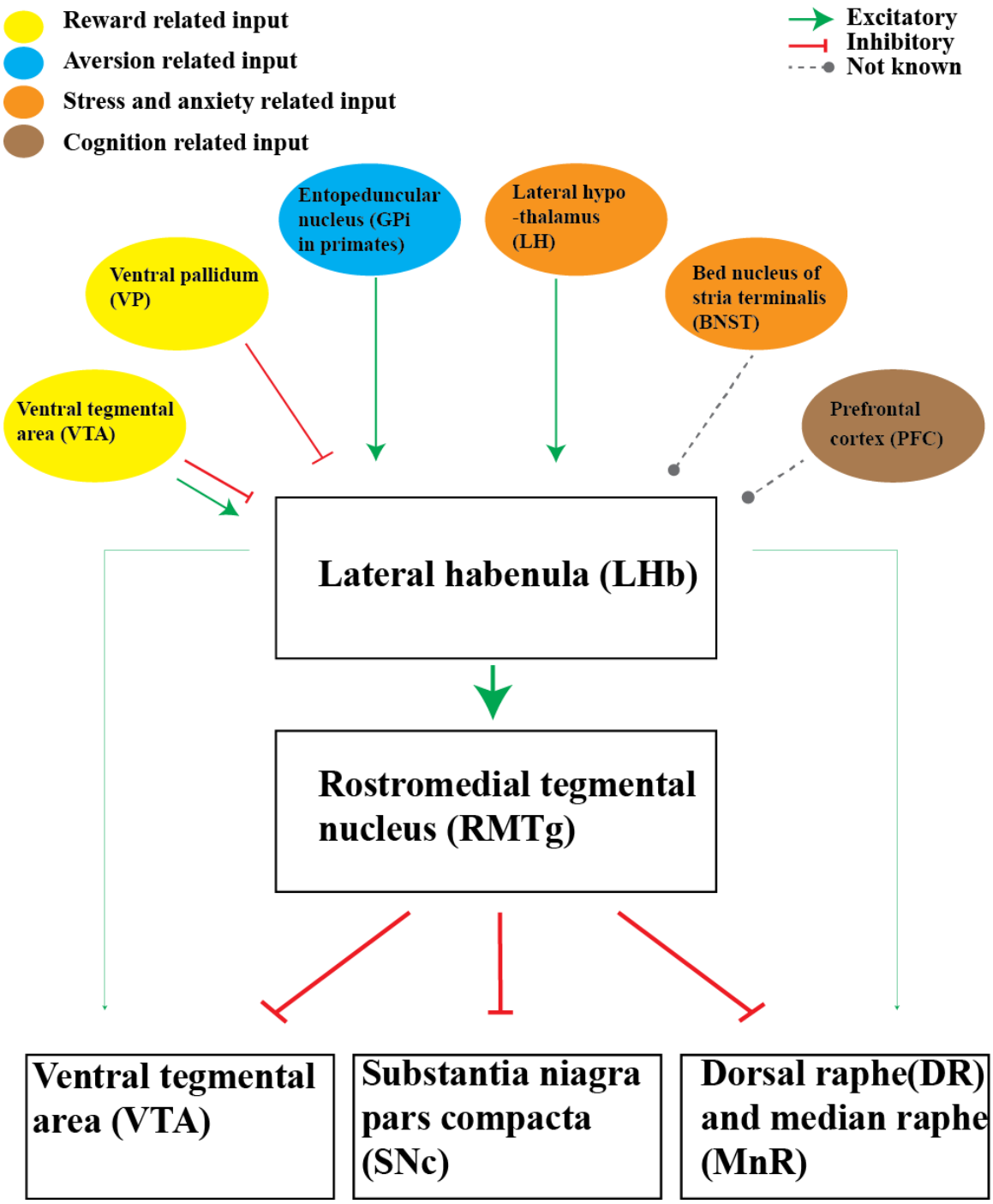
Reward related input
Aversion related input
Stress and anxiety related input
Cognition related input
Excitatory
Inhibitory
Not known
Entopeduncular nucleus (GPi in primates)
Lateral hypo -thalamus (LH)
Ventral pallidum (VP)
Bed nucleus of stria terminalis (BNST)
Ventral tegmental area (VTA)
Prefrontal cortex (PFC)
Lateral habenula (LHb)
Rostromedial tegmental nucleus (RMTg)
Ventral tegmental area (VTA)
Substantia niagra pars compacta (SNc)
Dorsal raphe(DR) and median raphe (MnR)

Table 1.1 LHb-RMTg pathway in drug-directed behaviors. From left to right, columns indicate the drug of abuse, effect of the drug on LHb activity, effect of drug on RMTg activity, and the role of LHb and RMTg in different drug-associated behaviors.

Drug of abuse	Effect on LHb activity	Effect on RMTg activity	Role of LHb & RMTg in drug-related behaviors
Cocaine	• Acute cocaine self-administration causes fos activation in LHb (Zahm et. al, 2010). • In rat brain slices, cocaine depolarizes LHb neurons and accelerates spontaneous firing (Zuo et. al, 2013). • Cocaine potentiates EPSC's in LHb neurons selectively projecting to RMTg (Maroteaux and Mameli, 2012). • Cocaine causes biphasic response in LHb neurons with an initial inhibition followed by rebound excitation (Jhou et. al, 2013).	• Acute cocaine causes FosB induction in RMTg GABA neurons which project to VTA DA neurons (Kaufling.et al, 2010).	• RMTg and FR lesions abolish avoidance response to cocaine (Jhou et. al, 2013). • DBS of LHb reduces cocaine self-administration, cue and cocaine-induced reinstatement and accelerates extinction (Friedman et.al, 2010). • LHb lesions increase cocaine-seeking and delay extinction (Friedman et. al, 2010). • RMTg activation consolidates extinction memory of cocaine-seeking (Huff et. al, 2014).
Ethanol	• Ethanol reduces LCGU in LHb (Williams-Hemby et. al, 1994). • Ethanol accelerates LHb firing which is dependent on glutamate and D1R's in LHb (Zuo et. al, 2015).	• No change in RMTg FosB induction after acute ethanol (Kaufling et. al, 2010). • Ethanol increases spontaneous activity of RMTg neurons in a rat strain bred for ethanol aversion (Melis et. al, 2014)	• LHb lesions increase voluntary ethanol intake, ethanol-seeking, attenuate ethanol-induced CTA and stress-induced reinstatement of ethanol -seeking (Haack et. al, 2014). • LHb inactivation abolishes ethanol-induced CPA (Zuo et. al, 2015).
Opioids	• Not studied	• Morphine inhibits firing of RMTg neurons, also reduces duration of RMTg-mediated inhibition of VTA DA neurons (Lecca et. al, 2011).	• Increased *c*-fos in LHb during cue-induced reinstatement of heroin-seeking (Zhang et. al, 2005). • Mice more vulnerable to relapse to morphine have increased *c*-fos in LHb (Madsen et. al, 2012).
Nicotine	• Acute and chronic nicotine increases *c*-fos expression in LHb (Mathieu-Kia et. al, 1998).	• Acute nicotine stimulates firing rate of RMTg neurons (Lecca et. al, 2011).	• D3 antagonist in LHb attenautes cue-induced reinstatement of nicotine-seeking (Khaled et. al, 2014). • LHb lesions reduce nicotine-induced anxiety (Casarrubea et.al, 2015).

CHAPTER 2

LESIONS OF THE LATERAL HABENULA INCREASE VOLUNTARY ETHANOL CONSUMPTION AND OPERANT SELF-ADMINISTRATION, BLOCK YOHIMBINE-INDUCED REINSTATEMENT OF ETHANOL SEEKING, AND ATTENUATE ETHANOL-INDUCED CONDITIONED TASTE AVERSION

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Lesions of the Lateral Habenula Increase Voluntary Ethanol Consumption and Operant Self-Administration, Block Yohimbine-Induced Reinstatement of Ethanol Seeking, and Attenuate Ethanol-Induced Conditioned Taste Aversion

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Abstract

The lateral habenula (LHb) plays an important role in learning driven by negative outcomes. Many drugs of abuse, including ethanol, have dose-dependent aversive effects that act to limit intake of the drug. However, the role of the LHb in regulating ethanol intake is unknown. In the present study, we compared voluntary ethanol consumption and self-administration, yohimbine-induced reinstatement of ethanol seeking, and ethanol-induced conditioned taste aversion in rats with sham or LHb lesions. In rats given home cage access to 20% ethanol in an intermittent access two bottle choice paradigm, lesioned animals escalated their voluntary ethanol consumption more rapidly than sham-lesioned control animals and maintained higher stable rates of voluntary ethanol intake. Similarly, lesioned animals exhibited higher rates of responding for ethanol in operant self-administration sessions. In addition, LHb lesion blocked yohimbine-induced reinstatement of ethanol seeking after extinction. Finally, LHb lesion significantly attenuated an ethanol-induced conditioned taste aversion. Our results demonstrate an important role for the LHb in multiple facets of ethanol-directed behavior, and further suggest that the LHb may contribute to ethanol-directed behaviors by mediating learning driven by the aversive effects of the drug.

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Introduction

The lateral habenula (LHb) has been importantly implicated in learning driven by adverse outcomes. Neurons in the primate LHb are excited by negative stimuli, such as an aversive air puff or cues predicting the absence of reward [1,2]. Excitation of LHb neurons results in inhibition of dopamine neurons in the ventral tegmental area and substantia nigra pars compacta [3]. This inhibition is mediated by a disynaptic pathway in which excitatory afferents originating in the LHb synapse on GABAergic neurons in the rostromedial tegmental area (RMTg), which target and inhibit downstream dopaminergic neurons [4]. Supporting a role for the LHb-RMTg pathway in learning driven by undesirable outcomes, manipulations that increase firing in the LHb, in LHb efferents to the RMTg, or in the RMTg itself are both acutely aversive and cause aversive conditioning [5–11].

The positively reinforcing effects of drugs of abuse motivate further drug seeking, particularly in initial stages of drug use [12]. However, these drugs also have aversive effects that limit voluntary intake [13]. Recent studies have implicated the habenula in negatively regulating motivation for both nicotine [14] and cocaine [6,15], and provide evidence that habenular circuits mediate learning driven by the aversive effects of these drugs. Ethanol consumption results in aversive effects that include acute sedation and motoric impairment as well as delayed hangover effects [16,17]. Sensitivity to these aversive effects is associated with decreased voluntary ethanol intake in rodent models, as shown by the inverse correlation of ethanol-induced conditioned taste aversion (CTA) with voluntary ethanol consumption and preference [18,19]. Along these lines, increased ethanol intake in adolescent rats (compared to adults) is accompanied by decreased ethanol CTA [20]; moreover, CTA magnitude in individual adolescent rats is inversely related to subsequent voluntary ethanol

intake [16]. Together these results suggest an important role for the aversive effects of the drug in suppressing voluntary ethanol consumption. Importantly, these effects are likely to be clinically relevant, as decreased sensitivity to the effects of ethanol, including aversive effects, is predictive of higher levels of binge drinking and higher risk for development of an alcohol use disorder in human populations [21–23].

The neural circuitry through which the aversive effects of ethanol suppress voluntary consumption is not well defined. Evidence supporting a role for the LHb in learning driven by aversive outcomes, including those caused by other drugs of abuse, raises the possibility that this brain region could contribute to these suppressive effects. To begin characterizing the role of the LHb in ethanol-directed behaviors, we studied voluntary ethanol consumption, ethanol self-administration, yohimbine-induced reinstatement of ethanol seeking, and ethanol-induced CTA in LHb and sham-lesioned rats. Our results show that lesions of the LHb increase both voluntary ethanol intake and self-administration, block yohimbine-induced reinstatement of ethanol seeking, and attenuate a taste aversion conditioned by a single noncontingent ethanol injection.

Materials and Methods

Ethics statement

All procedures used were approved by the University of Utah Animal Care and Use Committee and carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Subjects

136 male Long-Evans rats (300–350 g at the time of receipt; Charles-River, Wilmington, MA,) were used in the present experiments. Rats were single-housed in Plexiglas tub cages and maintained on a 12 hour light/dark cycle. *Ad libitum* access to food and water was available throughout all experimental procedures. A summary of the experimental groups and the timeline of experimental procedures (described below) within each group is provided in Table 1.

Drugs

Ethanol (Decon Labs, King of Prussia, PA) solutions were prepared in filtered tap water to a concentration of 20% (v/v) for use in the intermittent ethanol access paradigm, and prepared in physiological saline to a concentration of 20% for the CTA experiment. Saccharin, quinine and yohimbine (Sigma Aldrich, St. Louis, MO) solutions were prepared in distilled water.

Electrolytic lesion of the LHb

Surgical anesthesia was induced and maintained with isoflurane (5% and 2%, respectively). The skull was exposed and burr holes drilled bilaterally above the LHb. Lesions were produced by passing current (0.5 mA, 10 s) through a stainless steel electrode (AM Systems, Sequim, WA) at two sites within each hemisphere that targeted anterior and posterior portions of the LHb. Coordinates for these sites (in mm from bregma) were: –3 and –3.7 posterior; 0.7 lateral; and –5.4 ventral. In sham-lesioned animals, electrodes were lowered to stereotaxic coordinates 1 mm dorsal to the LHb but no current was passed at the target site. Rats were allowed to recover for at least one week after surgery before experiments commenced.

Voluntary ethanol consumption

Voluntary ethanol consumption was measured using an intermittent ethanol access (IEA) two bottle choice paradigm [24–26]. Rats (n = 34; 17 sham and 17 lesioned) were given 24 hour access to 20% (v/v) ethanol and water in a two bottle choice paradigm in the home cage 3 times per week (Monday, Wednesday and Friday). Ethanol bottles were placed in home cages at 9 a.m. on access days. Ethanol and water bottle positions within each cage were alternated in successive drinking sessions to minimize the effect of side preferences. On days in which ethanol was not presented, *ad lib* water was available. IEA was provided for a minimum of 8 weeks (24 drinking sessions). Ethanol and water intake were measured by weighing bottles before and after each 24 hour access period. These measures were used to calculate ethanol intake (normalized to weight, g/kg/24 h) and preference (ethanol intake/total fluid intake). Body weight and food intake were measured weekly during IEA.

In a subset of sham and lesioned rats (n = 7 rats in each group; see Table 1), the timeline of ethanol intake over 24 hour access periods was investigated by measuring intake in the first hour of ethanol access (9–10 am), the remainder of the light cycle (10 am – 6 pm), the first hour of the dark cycle (6–7 pm), and the remainder of the 24 hour period (7 pm – 9 am). These measurements were

Table 1. Summary of experimental groups and timeline of procedures. Numbered experiments indicated the order in which experiments within each group were carried out.

Rat group	Number of rats		Experiments
	Sham	Lesioned	
1	7	7	1) Intermittent ethanol access
			2) 24 h timeline of ethanol intake
			3) Effects of abstinence on IEA intake
			4) Taste preference
2	10	10*	1) Intermittent ethanol access
			2) Operant ethanol self-administration, extinction, and reinstatement
3	7	8	1) Operant sucrose self-administration, extinction, and reinstatement
4	37	42	1) Ethanol conditioned taste aversion
5	4	4	1) Ethanol metabolism

* A single lesioned rat died during intermittent ethanol access; the 9 remaining lesioned rats were trained in operant ethanol self-administration.

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carried out in rats that had IEA for 9 weeks (27 drinking sessions). In the same group of rats, the stability of ethanol intake after a period of abstinence was investigated. After an initial period of 10 weeks of IEA, rats were subjected to nearly 7 weeks (46 days) without ethanol access. IEA was then restored for two additional weeks to allow comparison of ethanol intake before and after abstinence.

Taste preference: two-bottle choice for saccharin and quinine solutions

Preference for saccharin solutions (0.005, 0.01, 0.05, 0.1, 0.5, 1 and 5 mM concentrations) and aversion to quinine solutions (0.0003, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3 mM) were assessed using a two-bottle choice paradigm in the home cage in a subset of sham and lesioned rats (n = 7 rats in each group). In this paradigm, a single bottle of tastant was made available for 48 hours in the home cage, concurrent with a single bottle of water; intake of the tastant and water were measured every 24 hours, and bottle positions were switched after the first 24 period. Intake during the two 24 hour periods was averaged to produce a single measure of consumption for each tastant concentration. Tastants were presented in sequential order of increasing concentration in consecutive 48 hour intervals. Intake during access to the quinine concentration series was measured first, followed by intake during presentation of the saccharin concentration series. To investigate the timeline of saccharin consumption over 24 hour access periods, intake of highly preferred 5 mM saccharin was measured during the four intervals in which ethanol intake was measured: 9–10 am, 10 am–6 pm, 6–7 pm, and 7 pm–9 am.

Operant responding for ethanol

After a minimum of two months home cage access to 20% ethanol in the IEA paradigm, rats (n = 10 sham and 9 lesioned rats) were trained to self-administer 20% ethanol. Operant chambers (Med Associates, St. Albans, VT) were equipped with a central reward receptacle flanked by retractable levers, with illuminated cue lights over each lever. Operant chambers were enclosed in sound attenuating chambers equipped with fans that increased ventilation and provided masking noise. Ethanol was delivered to the reward receptacle via a programmable syringe pump.

In an initial overnight session, rats were trained on an FR1 schedule to respond for 20% ethanol. In this overnight session, only the active lever was presented to facilitate learning. Each lever press caused the active lever to retract, the associated cue light to extinguish, and resulted in immediate delivery of 0.1 mL 20% ethanol into the reward receptacle. After 5 seconds, the active lever was extended and the cue light illuminated. After the initial overnight session, rats were trained in 1 hour sessions three times per week (Monday, Wednesday, Friday) using an FR1 schedule in which only the active lever was available. After 2 weeks, rats progressed to an FR3 schedule in which the session length was decreased to 30 minutes and the inactive lever was introduced. Lever presses on the inactive lever were recorded but had no programmed consequences. Responses in this paradigm were measured over two weeks before extinction training began (below).

Extinction and reinstatement of ethanol seeking

During extinction sessions, the syringe containing 20% ethanol was removed from the syringe pump, and thus active lever presses no longer resulted in ethanol delivery. In all other respects, the extinction paradigm was identical to the final operant response paradigm described above, including presentation of visual and auditory cues (cue light extinguishment, lever retraction, and syringe pump activation). Extinction sessions were run on alternate weekdays for four successive sessions before testing for yohimbine-induced reinstatement.

Reinstatement was studied by administration of the α2 receptor antagonist yohimbine (2 mg/kg, IP) or vehicle solution (distilled water) 30 minutes prior to testing in extinction sessions. Because yohimbine reliably induces multiple reinstatements of ethanol seeking [27], reinstatement in each animal was measured after each of two yohimbine injections to minimize variability in behavioral results. During reinstatement testing, rats were first tested for operant responding in an extinction session after injection of the vehicle solution. This was followed by a rest day (no injection or operant session). The next day, reinstatement of ethanol seeking after yohimbine injection was tested. Two additional extinction sessions followed, and then an identical testing schedule (vehicle injection, rest day, yohimbine injection) was carried out. For each animal, responses were averaged across each of the two drug administrations to yield a single measure of operant responding after vehicle administration and after yohimbine administration.

Sucrose: Operant responding, extinction and reinstatement

Fifteen ethanol-naive rats (7 sham and 8 lesioned rats) were trained to operantly self-administer 2% sucrose. With the exception of the use of sucrose as a reinforcer, the paradigm used was identical to the final operant paradigm used in ethanol self-administration (30 minute FR3 task with both active and inactive levers available). Training in this group was similar to that described for ethanol self-administration above, and began with an initial overnight training session (FR1, no inactive lever). In five subsequent sessions, the response requirement was maintained at FR1 but the session duration was shortened to a single hour. Finally, rats in this group progressed to the final paradigm (FR3, half hour duration, inactive lever introduced) for ten successive sessions.

Thereafter, extinction followed by reinstatement sessions were carried out. The extinction paradigm was identical to that described for extinction of ethanol seeking. A total of seven successive extinction sessions were run in this experiment. This increase (over four sessions used in extinction of ethanol seeking) was incorporated because response rates for sucrose self-administration were substantially higher than those occurring during ethanol self-administration. Yohimbine-induced reinstatement was tested in this experiment in a manner identical to that described above in studies of ethanol reinstatement.

Conditioned taste aversion

A total of 79 ethanol-naïve animals (37 sham and 42 lesioned male Long-Evans rats) were included in the CTA experiment. After lesion surgery, all rats were single housed and allowed at least one week recovery in home cages. Thereafter, rats were habituated to handling for two days before beginning the CTA experiment. Throughout the experiment, rats were maintained with *ad lib* access to both food and water. We avoided water deprivation often used in CTA paradigms because dehydration causes anorexia [28]. Food deprivation has been shown activate LHb neurons [29,30], raising the concern that water deprivation and associated dehydration-induced anorexia might differentially affect motivation in sham and LHb-lesioned rats. To induce consumption, a highly palatable supersaccharin solution (0.125% saccharin + 3% glucose) was used as the conditioned stimulus.

The first day of the CTA experiment, rats were given 30 minutes home cage access to supersaccharin. Rats were then assigned to saline or ethanol injection groups, with mean supersaccharin consumption matched between groups. Rats in the resulting four groups (LHb lesion/sham x saline/ethanol injection) were immediately injected with either ethanol (0.7 g/kg of 20% ethanol, IP) or saline (volume matched to ethanol injections) and returned to their home cages. The ethanol dose used was based on pilot studies showing it produced reliable but not saturating CTA. On each of the next six days, rats again received a single period of 30 minutes home cage access to the supersaccharin solution and consumption was measured. Supersaccharin access sessions, including the initial session, occurred between 4 and 6 pm daily.

Measurement of blood ethanol concentration

Blood ethanol concentration (BEC) was measured after voluntary ethanol intake in the IEA paradigm and operant self-administration sessions. BECs after ethanol consumption in the IEA paradigm were measured from tail vein blood collected after the first 30 minutes of ethanol access, the interval during which previous results [31] and our own measurements suggested intake rates were highest. BECs were measured in rats that had received 14 weeks of IEA. BECs after operant responding for ethanol were measured in tail vein blood collected immediately after 30 minute self-administration sessions.

To determine if LHb lesion altered ethanol metabolism, we measured BECs after noncontingent injection of ethanol (1 g/kg 20% ethanol, IP) in a separate group of ethanol-naïve rats (4 sham and 4 lesioned).

Blood samples were collected into heparinized capillary tubes at 15, 30, 60, 120 and 180 minutes after injection. For all BEC measurements, blood plasma was isolated from samples by perchloric acid precipitation and brief centrifugation (2000 rpm, 5 m). BEC was measured using the NAD-NADH enzyme spectrophotometric method [32,33].

Analysis of lesions

Rats were deeply anesthetized with pentobarbital and perfused with physiological saline followed by 4% formaldehyde. Brains were cryoprotected and sectioned in 50 µm slices. Sections were mounted and Nissl stained. Damage to the LHb was localized by comparison to a reference atlas [34] by an observer blind to behavioral results. Two lesioned rats died during or shortly after experimental procedures and lesion sites were not analyzed in these animals.

Though damage was largely confined to the LHb, some lesion sites encroached upon surrounding structures, including the medial habenula (MHb). To determine if damage to this structure contributed to voluntary ethanol intake in the IEA paradigm, we quantified the extent of this damage in each lesioned animal. The damage to the MHb in each hemisphere was estimated by visual inspection, and was scored as 0% (no damage), 25%, 50%, 75%, or 100% (complete ablation). Scores for each hemisphere were averaged to produce a single estimate of damage to the MHb within each rat. Rats were divided into high and low damage groups by performing a median split of this data, and ethanol intake in the IEA paradigm was then compared between groups.

Statistical analysis

Voluntary ethanol intake, escalation of ethanol intake, taste preference, and reinstatement of ethanol and sucrose seeking were analyzed using two-way repeated measures ANOVA. All analyses included lesion (sham or LHb) as one factor. The second factor consisted of IEA drinking session (voluntary ethanol intake); time interval (escalation of intake); tastant concentration (taste preference); or drug (reinstatement experiments; yohimbine or vehicle treatment). Extinction of ethanol-self administration and ethanol-induced CTA were analyzed using two- (factors of lesion and time) and three-way (factors of lesion, drug and time) ANCOVA, respectively. Baseline ethanol self-administration and supersaccharin intake were used as covariates in these analyses, respectively. Where appropriate, Holm-Sidak posthoc tests were used. BECs and operant self-administration were analyzed using Pearson's correlation test and t-tests.

Results

Voluntary ethanol consumption

Intermittent home cage access to 20% ethanol (IEA) in a two bottle choice paradigm resulted in steady escalation of ethanol consumption in both sham and lesioned animals (Fig. 1A; main effect of drinking session, $F(23, 728) = 28.2$, $p<0.001$). However, intake in the lesioned group diverged from that in the sham group after the first week (drinking sessions 1–3), escalated more rapidly over the course of the next several weeks, and plateaued at higher stable levels of ethanol intake (main effect of lesion, $F(1, 32) = 9.3$, $p<0.01$; significant interaction of lesion x drinking session, $F(23, 728) = 3.6$, $p<0.001$). Differences in ethanol consumption between sham and lesioned animals reached significance during the seventh drinking session and remained significant from the tenth through the final drinking session ($p<0.05$, posthoc tests). Ethanol consumption in the final drinking session averaged 4.1 ± 0.5 and 6.0 ± 0.5 g/kg/24 h for sham and lesioned animals, respectively. Differences in these normalized measures of ethanol intake were due solely to different levels of ethanol consumption, as mean rat weight did not differ between the two groups during IEA (data not shown; no significant effect of lesion on body weight, $F(1, 32) = 0.6$, NS; and no significant interaction of lesion x time, $F(7, 224) = 0.7$, NS; also no significant effect of lesion on food intake $F(1, 8) = 0.0$, NS; and no significant interaction of lesion x time, $F(8, 96) = 0.9$, NS). Similar differences were apparent in measures of ethanol preference (Fig. 1B), which averaged $43\pm5\%$ and $60\pm5\%$ in sham and lesioned animals, respectively, in the final drinking session (main effect of session, $F(23, 728) = 32.2$, $p< 0.001$; main effect of lesion, $F(1, 32) = 11.0$, $p<0.01$; significant interaction of lesion x session, $F(23, 728) = 2.9$, $p<0.001$). Posthoc testing indicated that differences between sham and lesioned rats were significant in drinking sessions 5, 7–8, and 10–24 ($p<0.05$, posthoc tests).

Water consumption in both groups decreased progressively over the course of drinking sessions (Fig. 1C; main effect of session, $F(23, 736) = 26.7$, $p<0.001$). Intake in lesioned rats was significantly lower than that in sham rats (main effect of lesion, $F(1, 32) = 5.1$, $p<0.05$; significant interaction of lesion x session, $F(23, 736) = 1.7$, $p<0.05$). Differences in water intake reached significance first in the 14^{th} drinking session and were intermittently significant thereafter ($p<0.05$, posthoc tests). Total fluid intake (water plus ethanol consumption) did not differ between the two groups (Fig. 1D; no significant effect of lesion, $F(1, 32) = 0.62$, NS; no significant interaction of lesion x session, $F(23, 736) = 1.2$, NS).

To determine if differences in voluntary ethanol consumption resulted in different blood ethanol concentrations (BECs), we measured ethanol concentration in tail vein blood collected after the first 30 minutes of ethanol access in the IEA paradigm. BEC measures were significantly correlated with ethanol consumption occurring during this period for both sham and lesioned rats (Fig.

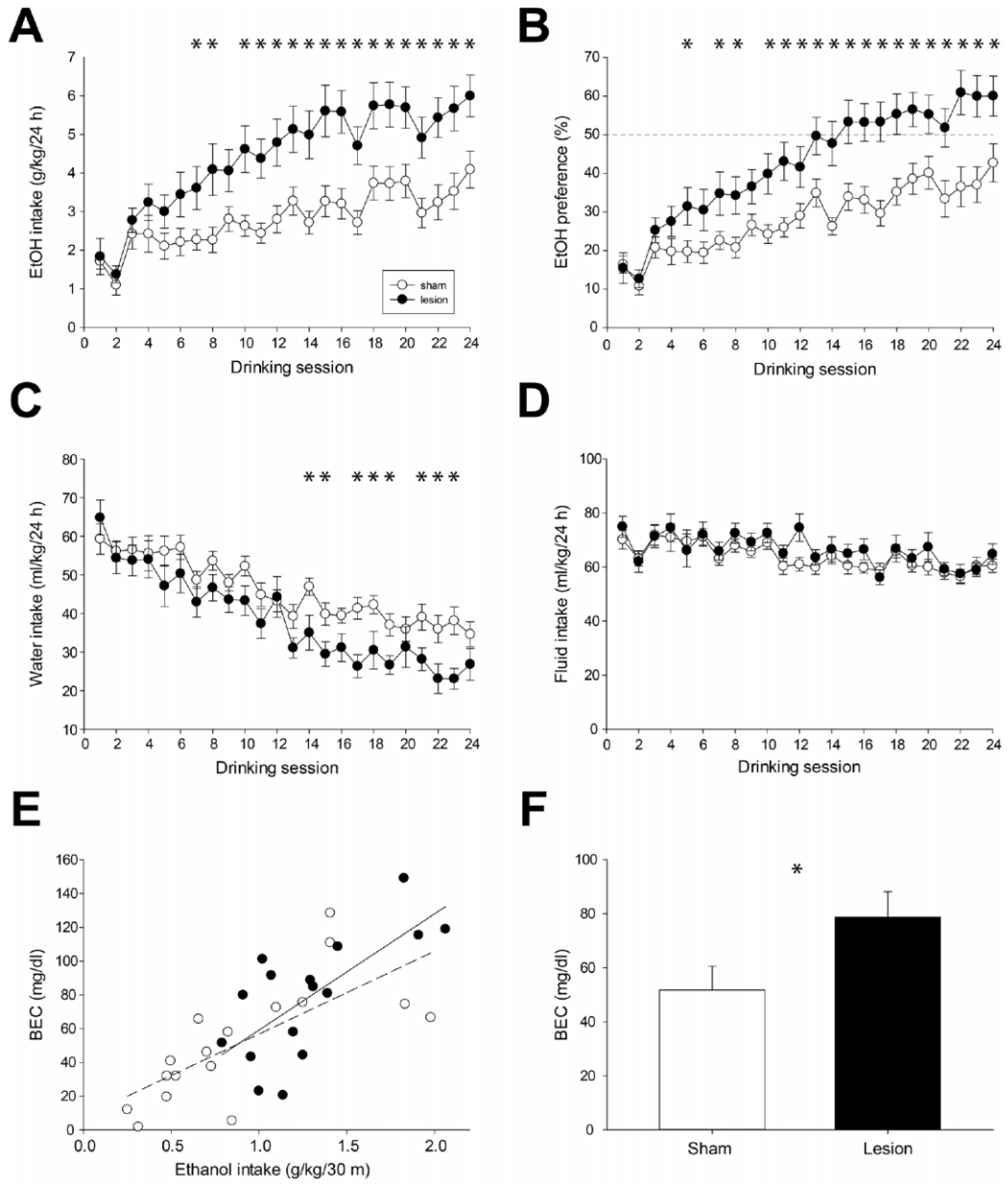

Figure 1. Voluntary ethanol consumption during intermittent access. (A) Lesioned rats consumed significantly more 20% ethanol over the course of 2-bottle choice drinking sessions (3 sessions/week for 8 weeks, 24 sessions total). Symbols indicate mean ethanol intake ± SEM. In this figure and Figures 2–5, filled symbols indicate lesioned animals, open symbols indicate sham animals, and asterisks indicate significant differences ($p<0.05$). (B) Alcohol preference in lesioned rats was significantly higher than that in sham rats. (C) Water intake decreased progressively in both groups, and rats in the lesioned group consumed significantly less water starting in the 5th week (14th session) of the paradigm. (D) Total fluid intake (water + ethanol) did not differ significantly between the two groups. (E) Blood ethanol concentrations (BECs) were significantly correlated with voluntary ethanol intake for both groups. Broken line indicates linear fit for sham group; solid line indicates linear fit for lesioned group. (F) Mean (± SEM) BEC measured after the first 30 minutes of 20% ethanol access was significantly higher in lesioned vs. sham rats ($p<0.05$).
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1E; $r^2 = 0.49$ and 0.52 for lesioned and sham rats respectively, $p < 0.01$ for each group). Lesioned rats consumed more ethanol during this period (mean ± SEM of 1.3±0.1 vs. 0.9±0.1 g/kg, $p<0.05$) and reached significantly higher BECs (Fig 1F; 79±9 vs. 52±9 mg/dl, $p<0.05$).

To determine if LHb lesion altered ethanol metabolism, we measured BECs at various time points after a single ethanol injection (1 g/kg 20% ethanol, IP; BEC measured 15, 30, 60, 120, and 180 m after injection) in a separate group of previously ethanol-naïve rats (n = 4 sham and 4 lesioned). BECs in these groups did not differ at any time point after ethanol injection (Table 2; no effect of lesion, $F(1, 6) = 0.04$, NS; no interaction of time x lesion, $F(6, 24) = 0.9$, NS), suggesting that lesion of the LHb had no effect on ethanol metabolism.

Average ethanol consumption in the first week did not differ between the two groups, though the lesioned group drank slightly more (Fig 2A; 2.1±0.3 vs. 1.7±0.2 g/kg/24 h in the lesioned and sham groups respectively, NS). By comparison, ethanol intake in the lesioned group was substantially higher than that in the sham group after eight weeks of IEA (Fig. 2B; 5.9±0.5 vs. 3.8±0.4 g/kg/24 h in the lesioned and sham groups respectively, $p < 0.01$). This higher level of intake was achieved by a more rapid escalation of intake in the lesioned group that persisted for approximately the first 5 weeks of ethanol access before intake levels plateaued thereafter (Fig. 1A). Analysis of the slope of increasing ethanol intake in these two intervals (first 5 weeks [sessions 1–15] vs. the last 3 weeks (sessions 16–24) of IEA) showed that the slope was significantly higher in the lesioned group during the first 5 weeks of IEA, but not thereafter (Fig. 2C; significant interaction of lesion x interval, $F(1, 32) = 7.9$, $p<0.01$; $p<0.05$, posthoc comparing slope of ethanol intake for sham vs. lesioned rats in sessions 1–15). Following the initial period of rapid escalation of ethanol intake, the rate of change in ethanol intake did not differ in lesioned and sham rats (no significant difference between sham and lesioned groups in sessions 16–24, posthoc NS).

To examine the stability of differences in ethanol consumption between the sham and lesioned rats, a subset of rats (n = 7/group) were allowed an initial 10 weeks of IEA, followed by nearly 7 weeks of abstinence (46 days), and then two additional weeks of IEA. Rats were maintained in their home cages during the period of abstinence with *ad lib* food and water supplied as usual. Analysis of 20% ethanol intake showed that lesioned rats consumed more ethanol (Fig. 2D-E; main effect of lesion, $F(1, 12) = 6.5$, $p<0.05$), and that this difference was stably maintained before and after the period of abstinence (sham rats – 4.8±1.0 before and 4.4±0.7 g/kg/24 h after; lesioned rats – 7.2±0.8 before and 6.7±0.7 g/kg/24 h after; no significant effect of time, $F(1, 12) = 3.6$, NS; no significant interaction of lesion x time, $F(1,12) = 0.01$, NS).

Taste preference

Gustatory function contributes to voluntary ethanol intake [35] and habenular lesions have been reported to alter bitter taste aversion [36]. We therefore investigated bitter and sweet taste preference in home-cage two bottle choice experiments in sham and lesioned rats. Quinine preference decreased as a function of concentration in both groups of rats (Fig. 3A; main effect of quinine concentration, $F(6, 12) = 58.7$, $p<0.001$), but did not differ between sham and lesioned rats (no significant effect of lesion, $F(1, 12) = 0.3$, NS; no significant interaction of concentration x lesion, $F(6, 72) = 0.3$, NS).

Saccharin preference increased as a function of increasing concentration (Fig. 3B; main effect of saccharin concentration, $F(6, 12) = 54.6$, $p<0.001$). There was no significant difference between sham and lesioned groups (no significant effect of lesion, $F(1, 12) = 2.4$, NS). Preference for saccharin appeared to be higher at highly preferred saccharin concentrations (0.5 mM and above) but this did not reach significance (no significant interaction of lesion x saccharin concentration, $F(6, 72) = 1.5$, NS). Ceiling effects may have obscured differences, however, as preference in both groups was near maximal at concentrations of 0.5 mM saccharin and above. Inspection of the volume of saccharin consumed showed that saccharin intake was significantly higher for lesioned rats at preferred concentrations (Fig. 3C; significant interaction of saccharin concentration x lesion, $F(6, 72) = 5.9$, $p<0.001$). Lesioned rats consumed significantly more than sham rats at saccharin concentrations of 0.5 mM and above ($p<0.05$, posthoc tests).

To further investigate elevated saccharin and ethanol intake in lesioned animals, we studied the timing of 5 mM saccharin and 20% ethanol ingestion during 24 hour periods of home cage access. The distribution of saccharin intake across the 24 hour cycle was similar for sham and lesioned rats, with both groups consuming saccharin at highest rates during the first hour of the dark cycle (Fig. 3D, left panel; main effect of time, $F(1, 12) = 57.0$, $p<0.001$; drinking rate during 1st hour of dark cycle [6–7 pm] significantly higher than that occurring during all other intervals, $p<0.05$, posthoc). Though lesioned rats consumed more saccharin than sham rats in every time period except the first hour of access, this difference did not reach statistical significance (no main effect of lesion, $F(1, 12) = 3.2$, NS; and no significant interaction of lesion x time, $F(3, 36) = 1.2$, NS). By contrast, lesioned rats consumed significantly more ethanol specifically during the first hour of ethanol access (Fig. 3D, right panel; significant interaction of lesion x time, $F(3, 36) = 2.9$, $p<0.05$; lesioned vs. sham intake in 1st hour of access, $p<0.05$, posthoc). Interestingly, these and previous results [31] suggest that the highest rates of voluntary ethanol intake leading to peak BECs occur within the first hour of access, and suggest that elevated ethanol intake in lesioned rats may be motivated by the pharmacological effects of the drug. Notably, rates of saccharin intake during the first hour of access were nearly identical in sham and lesioned animals.

Operant self-administration of 20% ethanol

A total of 19 rats (10 sham and 9 lesioned rats) with a history of IEA were trained to self-administer 20% ethanol on an FR3 schedule. Stable levels of responding (average of last three

Table 2. BECs (mg/dl) in ethanol naïve sham and lesioned rats following 1 g/kg IP ethanol injection.

	Time (minutes) after ethanol injection				
	15	**30**	**60**	**120**	**180**
Sham	129±15	118±7	108±9	76±11	31±11
Lesioned	118±18	108±14	109±11	77±4	38±9

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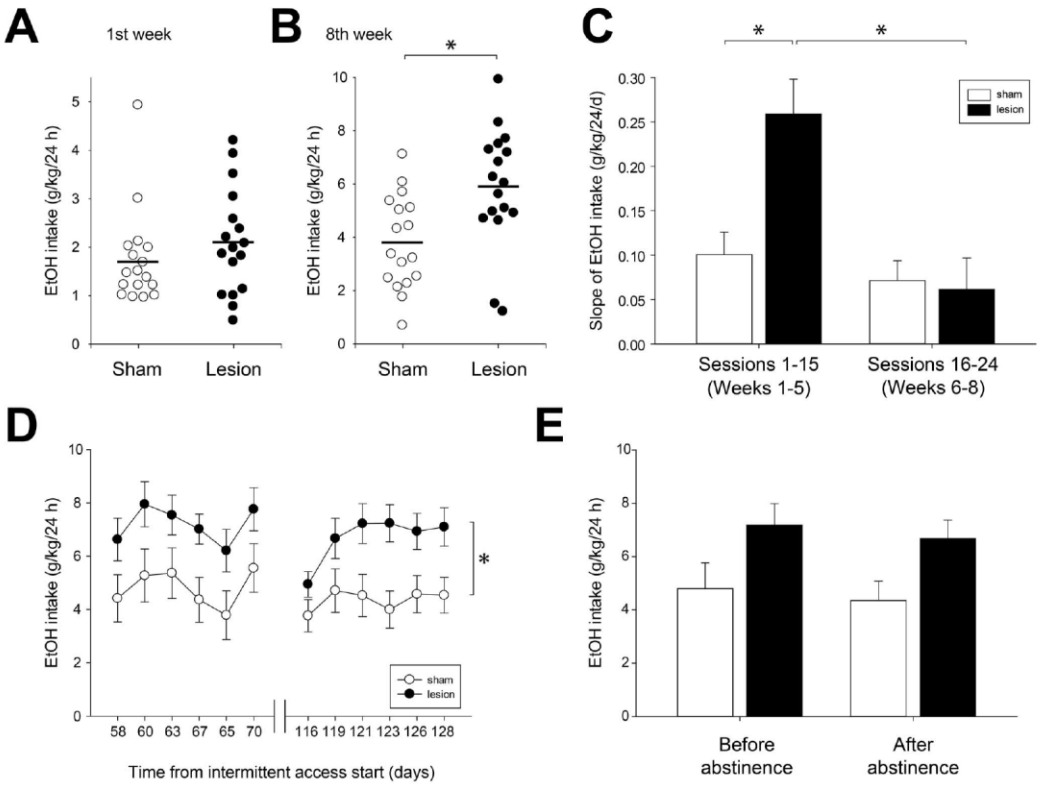

Figure 2. Escalation and stability of voluntary ethanol consumption. (A) Mean ethanol consumption did not differ between sham and lesioned groups in the first week (3 sessions) of IEA. Each symbol indicates the average ethanol intake for a single rat in the first week of IEA. Mean values of each group are indicated by horizontal bars. (B) Lesioned rats drank significantly more ethanol in the eighth week of IEA. (C) Lesioned rats escalated ethanol intake at higher rates than sham rats over the first 5 weeks of IEA (sessions 1–15), but not during the last 3 weeks of access (sessions 16–24). Bars graphs indicate the average slope of ethanol intake over the intervals shown. (D –E). Significant differences in ethanol consumption between groups were stably maintained after a period of abstinence. Rats were withdrawn from IEA for approximately 7 weeks (46 days), and then restored to IEA for an additional two weeks. Lesioned rats drank more than sham rats both before and after this period of abstinence. Asterisk (D) indicates significant main effect of lesion ($p<0.05$) on ethanol intake.
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rewarded sessions) were significantly higher in lesioned vs. sham rats (Fig. 4A; 87±15 vs. 50±10 lever presses for lesioned and sham rats, respectively; $t = 2.1$, $p<0.05$). In addition, levels of ethanol intake were higher in lesioned animals (Fig. 4B; 0.68±0.12 vs. 0.37±0.07 g/kg for lesioned and sham rats; $t = 2.3$, $p<0.05$). Increased lever pressing in lesioned rats was sustained for the duration of each operant session (Fig. 4C; main effect of lesion, $F(1, 17) = 5.9$, $p<0.05$; main effect of time, $F(14, 238) = 20.5$, $p< 0.001$; no significant interaction of lesion x time, $F(14, 238) = 0.6$, NS). BECs measured immediately after 30 minute operant response sessions revealed that lesioned rats achieved higher BECs during self-administration sessions (Fig. 4D; 42±12 vs. 13±4 mg/dl for lesioned and sham rats, $p<0.05$).

Operant self-administration of 2% sucrose

To determine if increased operant responding in lesioned rats occurred selectively for 20% ethanol, we studied self-administration of 2% sucrose. Sham and lesioned rats reached similar levels of stable operant responding for sucrose (Fig. 4E; 338±84 vs. 363±64 responses per session for sham and lesioned rats, respectively; $t = 0.2$, NS). In addition, the pattern of operant responding occurring within sessions did not differ between groups (Fig. 4F; significant main effect of time, $F(14, 182) = 29.4$, $p< 0.001$; but no significant effect of lesion, $F(1, 13) = 0.4$, NS; and no significant interaction of lesion x time, $F(14, 182) = 0.7$, NS).

Extinction and reinstatement of ethanol seeking

After rats reached stable levels of operant responding for ethanol, self-administration was extinguished over four successive extinction sessions. Lever pressing in the lesioned group was higher than that in sham rats in the first extinction session (Fig. 5A), but there was no significant interaction of lesion x extinction session ($F(3, 128) = 0.7$, NS) when ethanol self-administration rates were included as a covariate in the analysis (Fig. 5A, "Last rewarded session"). Mean response rates (± SEM) in the last extinction

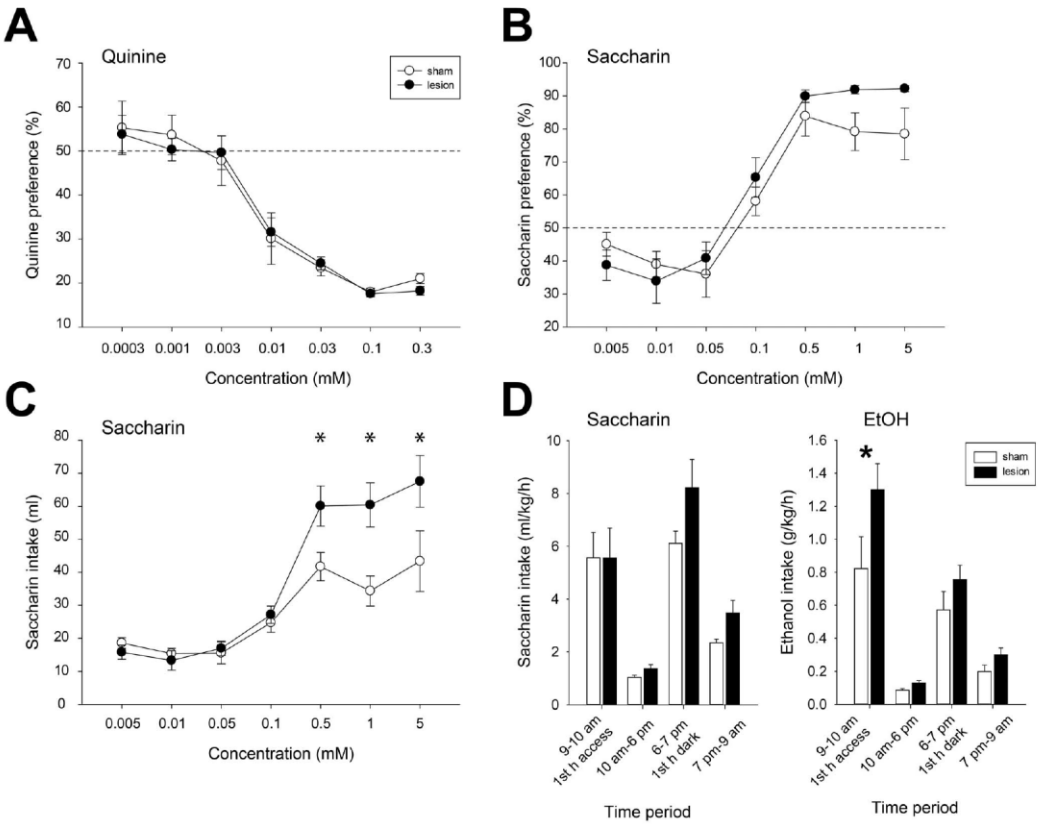

Figure 3. Taste preference. (A) Aversion to quinine did not differ between sham and lesioned rats. (B) Preference for saccharin did not significantly differ between sham and lesioned rats, despite quantitatively increased preference in lesioned rats at saccharin concentrations of 0.5 mM and above. (C) Saccharin intake was significantly higher in lesioned rats at concentrations of 0.5 mM saccharin and above. (D) Timeline of saccharin (left panel) and ethanol (right) intake over 24 hour sessions. The pattern of saccharin intake over 24 hours did not differ between sham and lesioned animals. Rates of intake were similar in the first hour of saccharin access and highest for both groups in the first hour of the dark cycle. By contrast, rates of 20% ethanol intake were higher in lesioned rats specifically during the first hour of access.
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session were 11±3 lever presses for lesioned rats and 14±4 for sham rats.

Reinstatement of ethanol seeking was tested by administration of yohimbine (2 mg/kg) or vehicle (distilled water) 30 minutes before testing in the extinction paradigm. Vehicle administration resulted in rates of operant responding very similar to those recorded in the final extinction session (Fig. 5B; 10±2 vs. 15±3 lever presses in lesioned and sham rats, respectively). Yohimbine caused a robust increase in operant responding in sham rats, but had no significant effect on responding in lesioned animals (18±8 vs. 47±9 lever presses in lesioned and sham rats, respectively; significant interaction of lesion x drug $F(1, 17) = 4.8$, $p<0.05$). Post hoc analyses showed that yohimbine administration increased lever pressing in sham rats relative to the lesioned group and relative to vehicle administration in the sham group ($p<0.05$, posthoc tests). Thus, lesion of the LHb blocked the ability of yohimbine to reinstate ethanol seeking.

Responses on the inactive lever did not differ between groups, nor were they affected by drug administration (Table 3; no effect of lesion, $F(1, 17) = 1.2$, NS; no effect of drug, $F(1, 17) = 0.8$, NS; no interaction of lesion x drug, $F(1, 17) = 3.6$, NS).

Extinction and reinstatement of 2% sucrose seeking

To determine if reinstatement deficits in lesioned animals were specific for ethanol seeking, we studied yohimbine-induced reinstatement of sucrose seeking in ethanol-naïve rats. After animals reached stable levels of 2% sucrose self-administration, responding was extinguished over seven extinction sessions. Lever press responses declined rapidly across extinction sessions (Fig. 5C; main effect of extinction day, $F(6, 78) = 34.7$, $p<0.001$) in both sham and lesioned rats (no effect of lesion, $F(1, 13) = 0.4$, NS; no interaction of lesion x day, $F(6, 78) = 1.0$, NS). Mean response rates in the final extinction session were 16±5 lever presses for lesioned rats and 4±1 for sham rats.

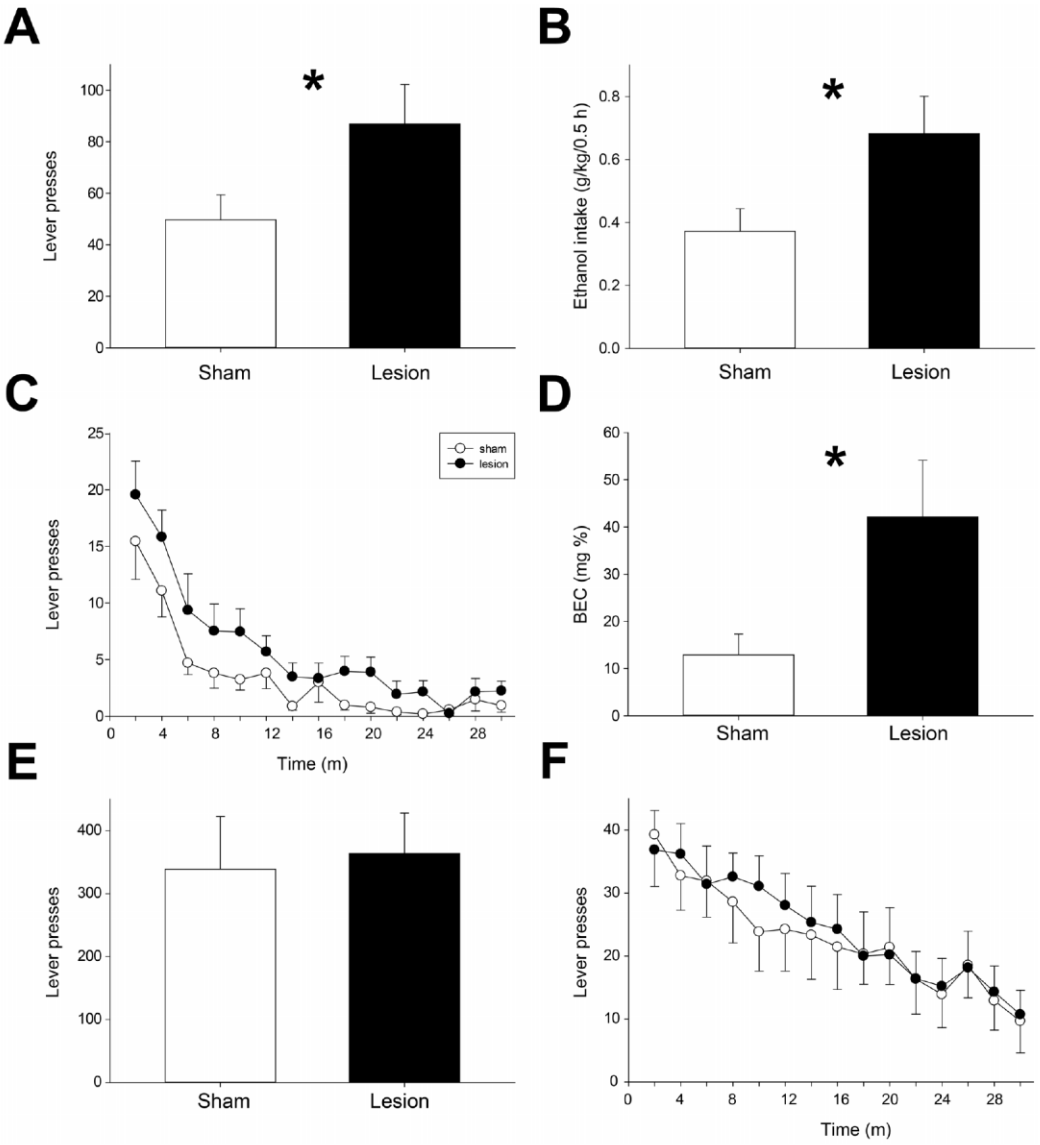

Figure 4. Operant self-administration of ethanol and sucrose. (A-B) Operant responding (A) for 20% ethanol was significantly higher in lesioned vs. sham rats, resulting in elevated levels of ethanol consumption (B). (C) Timing of lever pressing across 30 m self-administration sessions. Compared to sham animals, lesioned rats maintained elevated response rates for the duration of the session. (D) Operant self-administration resulted in significantly higher BECs in lesioned rats compared to sham rats. (E-F) Sham and lesioned rats showed similar levels of operant responding for 2% sucrose (E), and similar patterns of responding over the course of the 30 m session (F).
doi:10.1371/journal.pone.0092701.g004

Similar to the results seen for reinstatement of ethanol seeking, yohimbine reinstated sucrose seeking in sham but not lesioned rats (Fig. 5D; significant interaction of lesion x drug, $F(1, 13) = 6.5$, $p < 0.05$; also main effect of drug, $F(1, 13) = 6.4$, $p < 0.05$). Yohimbine administration resulted in a significant increase in operant responding in the sham group relative to operant responding after vehicle administration (19 ± 4 vs. 5 ± 2 lever presses after yohimbine and vehicle, respectively; $p < 0.05$, posthoc). A trend

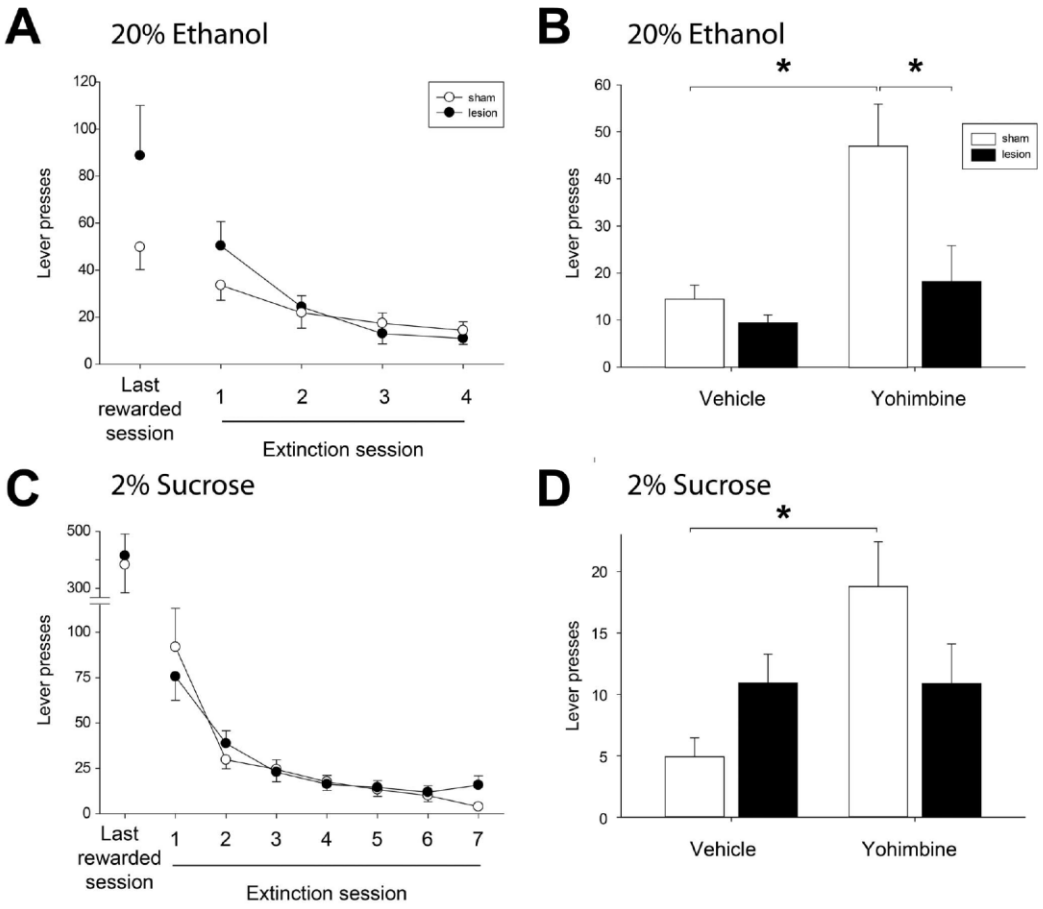

Figure 5. Extinction and reinstatement of ethanol and sucrose seeking. (A) The number of lever presses in lesioned and sham rats did not differ during extinction of operant responding for 20% ethanol. (B) Yohimbine administration reinstated ethanol seeking in sham but not lesioned rats. The number of lever presses by sham rats after yohimbine injection was significantly higher than that occurring after vehicle injection, and higher than lever presses performed by lesioned rats after yohimbine administration. Brackets indicate significant posthoc differences. (C) Sham and lesioned rats showed similar rates of extinction of responding for 2% sucrose. (D) Yohimbine administration induced reinstatement of sucrose seeking in sham but not lesioned rats.
doi:10.1371/journal.pone.0092701.g005

Table 3. Inactive lever presses following vehicle or yohimbine administration during reinstatement of ethanol or sucrose seeking.

	Group	Vehicle	Yohimbine
20% Ethanol	Sham	1.2±0.5	0.8±0.2
	Lesioned	0.9±0.4	2.1±0.7
2% Sucrose	Sham	1.1±0.4	1.3±0.4
	Lesioned	1.7±0.6	1.8±1.2

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toward increased operant responding in sham vs. lesioned rats was apparent after yohimbine administration ($p = 0.06$, posthoc test). Yohimbine did not reinstate sucrose seeking in the lesioned group (11±3 vs. 11±2 lever presses after yohimbine and vehicle, respectively; NS). Inactive lever presses did not differ between sham and lesioned rats, nor were they affected by drug administration (Table 3; no effect of lesion, $F(1, 13) = 0.2$, NS; no effect of drug, $F(1, 13) = 0.0$, NS; no interaction of lesion x drug, $F(1, 13) = 0.2$, NS).

Conditioned taste aversion

To determine if LHb lesion affected learning caused by ethanol's aversive effects, we examined ethanol-induced CTA in lesioned and sham rats. Rats in each group were injected with saline or ethanol (0.7 g/kg, IP) after an initial 30 minute period of supersaccharin access in the home cage. Ethanol administration conditioned an aversion to supersaccharin in both sham and lesioned groups as demonstrated by reduced supersaccharin intake after ethanol injection (Fig. 6; main effect of drug, $F(1, 73) = 40.9$, $p < 0.001$; also main effect of time, $F(3.2, 238.0) = 7.2$, $p<0.001$). However, the magnitude of this aversion was dependent on surgical treatment (significant interaction of drug x lesion, $F(1, 73) = 6.6$, $p<0.05$). Posthoc testing revealed that ethanol-induced CTA was attenuated in lesioned rats relative to sham animals, as supersaccharin consumption was significantly higher in lesioned vs. sham rats after ethanol injection (10.7±0.9 vs 7.7±1.0 ml, respectively; $p<0.05$). Lesioned and sham rats consumed similar amounts of supersaccharin on the first day after ethanol injection (Fig. 6, day 1), but lesioned rats appeared to recover from this initial aversion more rapidly than sham operated rats (consumption on days 2–6). However, there was no significant time-dependent difference in drug effects on sham vs. lesioned groups (no significant drug x lesion x time interaction; $F(3.2, 238.0) = 1.0$, NS). In lesioned and sham rats that received control saline injections, lesioned animals consistently consumed less supersaccharin than sham animals (days 1–6), but this did not reach statistical significance.

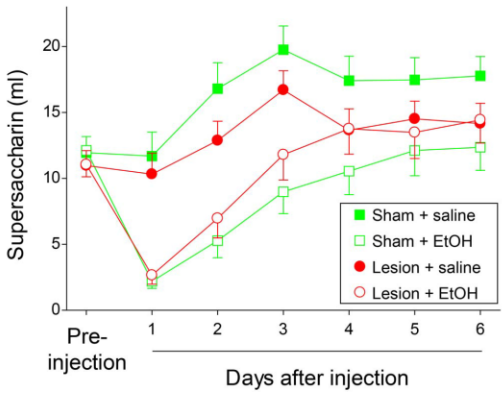

Figure 6. Ethanol induced conditioned taste aversion. LHb lesion attenuated the magnitude of a taste aversion conditioned by a single injection of ethanol. X-axis shows intake of supersaccharin; y-axis shows time of saccharin intake relative to injection in days. Squares and circles indicate sham- and LHb-lesioned rats, respectively. Filled and open symbols indicate saline and ethanol injection groups, respectively.
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Histological confirmation of lesions

Lesions were largely confined to the LHb (Figure 7), though they encroached upon neighboring structures, including the medial habenula (MHb). To determine if lesions of the MHb contributed to increased ethanol intake in lesioned animals, we quantified damage to this structure in lesioned rats tested in the IEA paradigm. Damage to the MHb ranged from a maximum of 50% (complete lesion of the MHb in one hemisphere) to 0%, and averaged 20±5%. Voluntary ethanol intake during IEA did not differ in rats with low vs. high MHb damage (5±2% vs. 33±5% damage, respectively, and ethanol intake of 6.3±0.4 and 5.8±0.9 g/kg/24 h in the last week of IEA in low and high damage groups, respectively; no effect of MHb damage, $F(1, 13) = 0.5$, NS; and no interaction of MHb damage x drinking session, $F(23, 292) = 0.7$, NS), suggesting that damage to this structure did not contribute to the results reported here.

Discussion

Our results show that the LHb plays an important role in controlling ethanol-directed behaviors. LHb lesion increased the rate at which rats escalated voluntary ethanol consumption in a two bottle choice paradigm, resulted in higher levels of maintained intake relative to sham rats both before and after a period of abstinence, and gave rise to higher BECs relative to sham animals. Operant self-administration of ethanol was also increased in lesioned rats, again resulting in higher BECs than those measured in sham animals. In addition, yohimbine-induced reinstatement of ethanol and sucrose seeking were blocked in animals with LHb lesions. Finally, our results show that ethanol CTA was significantly attenuated by LHb lesion. These results demonstrate an important role for the LHb in regulating ethanol-seeking and consumption and moreover show that LHb circuits contribute to learning driven by ethanol's aversive effects.

Effects of LHb lesion on ethanol intake and self-administration

LHb lesions did not acutely increase voluntary ethanol consumption, but rather increased the rate at which escalation of intake occurred, leading to higher sustained levels of ethanol consumption. Ethanol intake in the first week of the IEA paradigm was similar in sham and lesioned groups (Figs. 1A and 2A). Drinking levels in the two groups diverged gradually, with LHb-lesioned rats escalating their intake more rapidly than those in the sham group (Figs. 1A and 2C). Drinking in lesioned rats plateaued around the 6th week of IEA, at which time ethanol intake in lesioned rats was approximately 50% higher than that in the sham group. This difference in intake was maintained for the remainder of the IEA paradigm.

Changes in taste preference are unlikely to contribute to increased ethanol intake and self-administration in LHb-lesioned rats. Though lesions of the habenular complex have been reported to decrease quinine intake [36,37], we found that sham and lesioned rats showed nearly identical dose-dependent aversion to quinine (Fig. 3A). LHb lesion clearly increased consumption of preferred saccharin concentrations (Fig. 3C; concentrations of 0.5 mM and higher). However, patterns of elevated saccharin and ethanol intake differed, suggesting distinct physiological mechanisms underlying consumption of the two solutions (Fig. 3D). Saccharin consumption occurred at highest rates during the first hour of the dark cycle (6-7 pm). In contrast, rates of ethanol intake were highest for both groups during the first hour of access (9-10 am), and were significantly higher in the lesioned group exclusively during this period. Because drinking during this interval is likely to

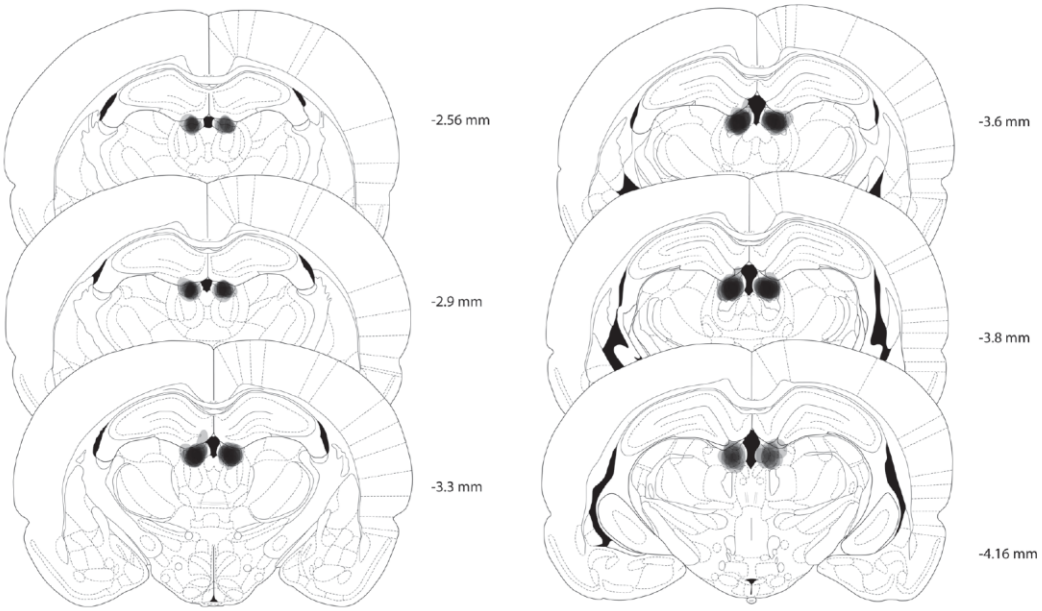

Figure 7. Lateral habenula lesions. Lesion sites were centered on the lateral habenula. Semi-transparent shading shows lesion sites overlaid for each rat, so that darkest areas indicate areas of greatest damage. In some rats, lesions extended to nearby structures (particularly including the medial habenula, medial thalamic areas, and the dentate gyrus). Numbers to the right of each section indicate anterioposterior position relative to bregma.
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result in the highest BECs [31], this result suggests that increased ethanol intake in lesioned rats during this interval may be motivated by the pharmacological effects of the drug.

Our findings of increased ethanol intake and self-administration in LHb-lesioned animals contribute to a growing body of evidence implicating the habenula in regulating drug seeking behaviors. Habenular circuits have been shown to play a central role in regulating learning driven by negative outcomes [6–11], and recent studies have shown that this function importantly extends to regulation of drug-seeking behaviors [6,14,15]. Electrical stimulation of the LHb accelerates extinction of cocaine self-administration and attenuates subsequent cocaine-induced reinstatement [15], consistent with a mechanism in which excitation of LHb neurons suppresses drug-seeking through aversive conditioning. Jhou and colleagues [6] have shown that cocaine induces delayed excitation in a subset of LHb and downstream RMTg neurons, and that optogenetic inhibition of RMTg firing specifically during this period of delayed excitation blocks cocaine-conditioned avoidance behaviors. In addition, studies of mechanisms regulating nicotine intake suggest an analogous role for circuits originating in the medial habenula in mediating aversion-dependent suppression of nicotine self-administration [14].

Increased ethanol intake and self-administration in LHb-lesioned animals may also arise through deficits in learning from aversive drug outcomes. Ethanol causes dose-dependent aversive effects that include nausea, sedation, motoric impairment and hangover effects [16]. These effects are thought to condition a learned aversion to ethanol that acts to decrease subsequent intake, as suggested by the inverse correlation of ethanol CTA and voluntary ethanol intake [18,19]. Our results show that lesion of the LHb causes attenuation of a taste aversion conditioned by a single noncontingent ethanol injection. LHb neurons are known to be excited by negative stimuli, including drug-induced negative stimuli [1,2,6]. Loss of an ethanol-induced aversive signal after LHb ablation could thus contribute to increased ethanol intake in lesioned rats. In this regard, our finding that LHb lesion did not acutely increase voluntary ethanol consumption, but rather increased the rate at which escalation of intake occurred, is suggestive of an impairment in ethanol-induced aversion learning. However, additional experiments are needed to determine if attenuated CTA in LHb-lesioned animals plays a causal role in increasing voluntary ethanol intake.

Effects of LHb lesion on extinction and reinstatement of ethanol and sucrose seeking

Given evidence of an important role for the LHb in aversive conditioning, lesion of this structure might be expected to impair extinction learning, particularly as lesion and stimulation of the LHb respectively impair and accelerate extinction of cocaine-seeking [15,38]. However, we found no extinction deficits in lesioned rats. Our results agree with those reported in a recent study of cocaine self-administration, in which operant responding in an initial extinction test was unaltered by acute pharmacological inactivation of the LHb [39]. These findings contrast with those of a recent study showing that LHb lesions block extinction learning in rats trained to self-administer cocaine [15]. Methodological differences (bilateral electrolytic lesions vs. unilateral excitotoxic lesion) and/or the drug tested could contribute to these divergent results. It is also possible that recovery of neural function may have contributed to intact extinction learning in our lesioned animals, as

operant responding in extinction was tested weeks after lesions were made.

The effects of LHb lesion were not specific to reinstatement of ethanol seeking, as yohimbine-induced reinstatement of sucrose seeking was also blocked in lesioned animals. Our results extend recent findings that pharmacological inactivation of the LHb blocks yohimbine-induced potentiation of cue-dependent reinstatement of cocaine-seeking, as well as attenuating yohimbine-induced anxiety-related behaviors [39]. Habenular neurons, particularly those in the medial subdivision of the LHb, are activated by an array of stressful stimuli, including foot shock, novel environments, restraint stress, food restriction and lithium chloride injection [29,30,40,41]. Habenula lesions impair stress-induced potentiation of prepulse inhibition, decrease avoidance learning tested under high stress conditions, and block the development of learned helplessness in response to inescapable shock [42–44]. Taken together, these results demonstrate an extensive role for habenular function in mediating stress-induced behavioral responses. These results may have clinical implications, as they raise the possibility that individual differences in LHb function could importantly contribute to vulnerability to stress-induced relapse of drug seeking in abstinent alcoholics.

In summary, our study provides novel evidence of an important role for the LHb in a number of ethanol directed behaviors. Our results show that LHb lesions increase voluntary ethanol intake and operant-self administration and block yohimbine-induced reinstatement of ethanol seeking. Further, LHb lesions attenuate ethanol CTA. These results are likely to have important implications for mechanisms underlying alcohol use disorders as studies in human volunteers show that vulnerability to developing an alcohol use disorder is inversely related to sensitivity to acute ethanol effects, including the aversive effects of the drug [21–23].

Author Contributions

Conceived and designed the experiments: SAT. Performed the experiments: AKH CS ALS MSS ST SAT. Analyzed the data: AKH CS SAT. Contributed reagents/materials/analysis tools: AKH CS SAT. Wrote the paper: SAT.

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CHAPTER 3

LESIONS OF THE LATERAL HABENULA DO NOT ALTER ACUTE AVERSIVE PROPERTIES OF ETHANOL

Abstract

I have shown previously that lesions of the LHb increase voluntary ethanol consumption and ethanol-seeking in rats. The attenuated ethanol-induced conditioned aversion in LHb-lesioned rats could be one of the mechanisms by which LHb lesions could increase ethanol-consummatory and -seeking behaviors. However, it is plausible that lesions of the LHb alter acute aversion to ethanol, which could be a potential alternate mechanism for increased ethanol intake seen in LHb-lesioned rats. I investigated the acute aversive properties of ethanol, such as motor impairment, sedation, and ethanol-withdrawal-induced anxiety in LHb-lesioned rats. My results show that lesion of the LHb does not significantly alter acute aversion to ethanol.

Introduction

Drugs of abuse, including ethanol, have both rewarding and aversive properties, both of which play an important role in ethanol intake (Verendeev and

Riley, 2013). Preclinical and clinical evidence suggest that sensitivity to aversive effects of ethanol can serve as a protective factor against excessive ethanol intake (Green and Grahame, 2008; King et al., 2011). Ethanol has aversive properties that include nausea, sedation, motor impairment, and hangover-like effects (Schulteis and Liu, 2006). Previous studies have shown that each of the acute aversive effects of ethanol can negatively regulate ethanol intake (Little et al., 1996; White et al., 2002; Doremus-Fitzwater and Spear, 2007).

I have previously shown that lesions of the LHb increase voluntary ethanol consumption and attenuate ethanol-induced CTA (Haack et al., 2014, Chapter 2). In agreement with these results, another recent study showed that inhibiting LHb activity abolishes place aversion conditioned by ethanol (Zuo et al., 2015). These results suggest that increased ethanol intake in LHb-lesioned rats (Haack et al., 2014) could result from an attenuation of ethanol-induced aversion learning. However, it was heretofore unclear if lesions of the LHb affect the acute aversive properties of ethanol described above, which could be an alternate mechanism contributing to the increased ethanol intake seen in LHb-lesioned rats. Thus, in the present study I examined the impact of LHb lesions on ethanol-induced motor impairment, ethanol-withdrawal-induced anxiety, and ethanol-induced sedation. My results support the conclusion that LHb lesions do not alter the acute aversive effects of ethanol, consistent with a primary role for impaired aversion learning in driving increased ethanol intake in LHb-lesioned rats (Haack et al., 2014).

Materials and methods

Subjects

Sixty male Long-Evans rats (300-350 g on receipt; Charles-River, Wilmington, MA) were used. Rats were single-housed in Plexiglas tub cages and maintained on a 12-hour (h) light/dark cycle. *Ad libitum* access to food and water was available at all times. All procedures occurred in the light cycle (12:12 h), with lights on at 6 AM unless otherwise stated. All procedures used were approved by the University of Utah Animal Care and Use Committee and carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* (8^{th} edition).

Drugs

Ethanol (Decon Labs, King of Prussia, PA) was prepared as a 20% v/v solution in physiological saline.

LHb lesions

Surgical procedures were conducted under isoflurane anesthesia (5% induction, 2% maintenance). Neo-Predef (a topical anaesthetic), penicillin (3 x 108 units/kg, i.m.), and buprenorphine (0.06 mg/kg, intraperitoneal (IP)) were also administered for analgesia and to prevent infection. Rats were placed in a flat-skull position in a stereotaxic apparatus, the skull exposed, and burr holes drilled above the target region. Bilateral electrolytic lesions were produced by passing current (0.5 mA, 20 sec) through a stainless steel electrode (AM

Systems, Sequim, WA) at the following coordinates: AP: -3.7 mm, ML: ±0.7 mm and DV: -5.6 mm, relative to bregma (Paxinos and Watson, 2007b). For sham lesions, the electrode was targeted to a site 1 mm above the LHb, and no current was passed. Rats were monitored daily for at least 1 week prior to behavioral procedures.

Rotarod and loss of righting reflex (LORR) tests were used to assess the acute effect of ethanol on motor function and sedation, respectively, and the elevated plus maze (EPM) was used to assess the effects on ethanol-withdrawal-induced anxiety. Individual rats were used in one, two, or all three of these experimental paradigms. For rats used in multiple paradigms, a rest period of at least 1 week was allowed between tests.

Rotarod

A rotarod apparatus (Accurotor Rotarod, AccuScan Instruments, Columbus, OH) was used to test acute ethanol effects on motor coordination and balance. A total of 28 (14 sham- and 14 LHb-lesioned) rats were tested on the rotarod after injection of ethanol. However, data from 3 LHb-lesioned rats were eliminated due to misplacement of the lesion, resulting in a final sample size of 14 sham- and 11 LHb-lesioned animals.

Prior to behavioral testing, rats were first habituated to the apparatus. During habituation sessions, rats were required to complete 5 training trials. For each trial, rats were placed on the rotating drum at a rotation speed of 4 rpm. The speed was then accelerated from 4 rpm to 40 rpm over 4.5 minutes. Rats were

required to remain on the drum for at least 2 minutes without falling, in order for a training trial to be completed. The next day, after one further training trial, rats were tested following an injection of 1.5 g/kg body weight of 20% v/v ethanol (IP). The length of time the rats remained on the drum was then recorded at 10, 20, 30, 60, 90, 120,150 and 180 minutes post-injection. Rats were returned to their home cages between trials.

Elevated plus maze

A total of 45 rats (23 sham- and 22 LHb-lesioned) were tested on EPM. However, data from 5 LHb-lesioned rats were eliminated from analyses due to misplacement, and 1 sham-lesioned rat was removed since it was a statistical outlier (3 S.D. away from the mean), resulting in a final sample size of 22 sham- and 17 LHb-lesioned rats.

The elevated plus maze (EPM) apparatus consisted of a plus maze with two open arms (50 cm length, 10 cm width, 1 cm high rail) and two enclosed arms (50 cm length, 10 cm width, 41 cm high wall). The two open arms were positioned opposite each other, at a height of 92 cm from the floor. Rats were habituated to the room and the apparatus. Twelve hours prior to testing (9 PM), rats were injected with 1.5 g/kg body weight of 20% v/v ethanol (IP) or an equivalent volume of saline, and were then returned to the home cages. The next morning (9 AM), rats were placed in the center of the EPM, facing an open arm. During tests, the position of each rat in the maze was recorded with an overhead camera, under low-light conditions (~10 lux), with illumination provided by a

single red light source. The criterion for arm entry was defined as all 4 paws in the arm of the maze. The time spent in the open and closed arms was recorded for 10 minutes. The apparatus was wiped with a damp cloth between animals.

Loss of righting reflex

A total of 37 rats (19 sham- and 18 LHb-lesioned) were tested for LORR. Data from 4 sham- and 1 LHb-lesioned rat were eliminated from consideration since they failed to lose their righting reflex within 10 minutes of injection. Also, data from another 5 LHb-lesioned rats were eliminated due to misplacement of the lesion, resulting in a final sample size of 15 sham- and 12 LHb-lesioned rats.

LORR and recovery of the righting reflex (RORR) protocols were based on previously published literature (Pian et al., 2008). Rats were first injected with 4 g/kg body weight of 20% v/v ethanol (IP) and then placed in a clean Plexiglas tub containing paper bedding. Every 20-30 seconds, each rat was placed on its back until it was unable to right itself within a 20-second interval. Righting was defined as all 4 paws making contact with the floor. The time between the injection and failure to right was recorded as the time to LORR. Time to RORR was identified as the time at which the rat had regained the ability to right itself twice across two successive 20-second periods. The time to RORR was recorded as the difference in time between the onset of LORR and the onset of the successful RORR period. Immediately after RORR, a blood sample was taken for blood ethanol concentration (BEC) analysis. For all BEC measurements, tail vein blood was collected; blood plasma was isolated from samples by perchloric acid

precipitation and brief centrifugation (2000 rpm, 5 minutes). BEC was then measured using the NAD-NADH enzyme spectrophotometric method (Weiss et al., 1993; Zapata et al., 2006).

Verification of lesions

Rats were deeply anesthetized with sodium pentobarbital (140 mg/kg) and transcardially perfused with saline, followed by 4% formaldehyde. The brains were removed, cryoprotected and then cut on a freezing microtome (45 µm thickness). Sections were mounted onto glass slides, dried and stained with cresyl violet before being dehydrated and cover slipped. Lesions were verified using a light microscope, and plotted on templates modified from a reference rat brain atlas (Paxinos and Watson, 2007b).

Statistical analyses

Lesion effects on LORR, RORR, and BEC at RORR were analyzed using t-tests. Rotarod results were analyzed using two-way repeated measures (RM) ANOVA (factors of lesion and time after injection). Performance on the EPM was analyzed using two-way ANOVA (factors of lesion and drug). JMP Pro 11 (SAS Institute Inc., Cary, NC) was the statistical software used to carry out analyses. Analyses were considered significant when $p<0.05$. Data are shown as mean ± S.E.M.

Results

Rotarod

Acute ethanol injection impaired rotarod performance, demonstrated by reduced latency to fall (Figure 3.1a; significant main effect of time, $F(4.4,100.3)=23.7$, $p<0.0001$). However, LHb lesion had no effect on rotarod performance (no significant main effect of lesion, $F(1,23)=0.6$, $p=0.45$; no significant interaction of lesion and time, $F(4.3,100.3)=0.4$, $p=0.84$).

Elevated-plus maze (EPM)

Ethanol withdrawal decreased the amount of time spent exploring open arms of the EPM (Figure 3.1b; significant main effect of drug, $F(1,37)=7.2$, $p<0.05$). Overall, LHb-lesioned rats spent more time exploring the open arms (significant main effect of lesion, $F(1,37)=5.5$, $p<0.05$), but ethanol injection had similar effects on sham- and LHb-lesioned groups (no significant interaction of lesion and drug; $F(1,37)=1.0$, $p=0.33$).

Righting reflex

LHb lesion had no significant effect on time to LORR (Figure 3.2a; $t=1.6$, $p=0.12$), time to RORR (Fig 3.2b; $t=-0.49$, $p=0.63$) or on BEC levels at RORR (Fig 3.2c; $t=-0.3$, $p=0.74$).

Histological confirmation of lesions for LHb

Lesions were largely confined to the LHb (Figure 3.3). There was some damage to the adjacent medial habenula (MHb) in some cases, as well as to the FR, the major output pathway of the LHb.

Discussion

In the current study, I investigated the effects of lesion of the LHb on the acute aversive effects of ethanol, including motor impairment, ethanol-withdrawal-induced anxiety, and sedation. My results demonstrate that lesion of the LHb does not significantly change ethanol-induced motor impairment and sedation or ethanol-withdrawal-induced anxiety; however, LHb-lesioned rats do have significantly lower basal levels of anxiety. I discuss the implications of these findings in more detail below.

I have previously shown that lesions of the LHb increase voluntary ethanol consumption and attenuate ethanol-induced CTA (Haack et al., 2014). Attenuation of ethanol-induced CTA suggests LHb lesion reduces rats' ability to learn from the aversive effects of ethanol intake. Decreased sensitivity to conditioning by aversive effects of ethanol is a candidate mechanism for mediating increased escalation of voluntary ethanol intake in LHb-lesioned animals (Haack et al., 2014). However, in the original study, I did not examine LHb lesion effects on behavioral responses to the acute effects of ethanol administration. Reduced sensitivity to ethanol's acute aversive effects (e.g., sedation, motor impairment, and hangover-like effects) could also potentially

contribute to increased ethanol intake. Reduced sedation, motor impairment and ethanol-withdrawal-induced anxiety have been noted in adolescent vs. adult rats (Little et al., 1996; White et al., 2002; Doremus-Fitzwater and Spear, 2007), and these traits have been hypothesized to contribute to higher levels of voluntary ethanol consumption in adolescent rats (Schramm-Sapyta et al., 2010). Also, humans who are less sensitive to the acute effects of ethanol, including aversive effects, are more likely to develop alcoholism (Schuckit et al., 1996; Schuckit et al., 2004; Schuckit et al., 2006). Together, these data suggest that motor impairment, sedation, and hangover-like effects, including ethanol-withdrawal-induced anxiety, can negatively regulate voluntary ethanol intake. Thus, in the present study, I investigated if lesions of the LHb alter ethanol's acute aversive physiologic effects.

Consistent with previous studies (Chuck et al., 2006), I found that ethanol caused significant motor impairments, evident in rotarod performance. However, the degree of impairment did not differ between sham- and LHb-lesioned groups (Figure 3.1a). Similarly, I found that LHb lesions did not significantly change ethanol-induced sedation and recovery from sedation: the time to LORR (Figure 3.2a), time to RORR (Figure 3.2b), and BEC levels at RORR (Figure 3.2c) did not differ between groups.

Ethanol withdrawal causes anxiety-like behavior, apparent as decreased time spent in open arms on the elevated-plus maze (Doremus-Fitzwater and Spear, 2007). In agreement with previous literature (Zhang et al., 2007), I found that ethanol withdrawal increased anxiety; however, LHb lesion did not change

the magnitude of this effect (Figure 3.1b). Interestingly, this finding contrasts with those from a recent study which reported that LHb lesions reduce nicotine-induced anxiety (Casarrubea et al., 2015). Methodological differences, including the drug tested (ethanol vs. nicotine) and the apparatus used (EPM vs. hole-board), could contribute to these divergent results.

Overall, LHb lesion did reduce anxiety as measured in the EPM, consistent with previous findings showing that LHb inactivation reduces anxiety under basal as well as heightened stress conditions (i.e., after yohimbine injection) (Gill et al., 2013). Whether a lower anxiety level seen in LHb-lesioned rats contributes to increased ethanol consumption (Haack et al., 2014) is subject to debate. Studies looking at correlations between innate anxiety and voluntary ethanol consumption in both rodent models as well as humans have yielded conflicting results. Some studies support a positive correlation between innate anxiety and voluntary ethanol consumption (Stewart et al., 1993; Spanagel et al., 1995), while others suggest, somewhat unexpectedly, that innate anxiety and voluntary ethanol consumption are negatively correlated (Rohsenow, 1982; Moller et al., 1997; Henniger et al., 2002). Additional studies examining anxiety vs. voluntary intake, and the effects of LHb lesion on this relationship, are therefore required. Regardless, my current results suggest that LHb plays little role in mediating the acute aversive, use-limiting effects of ethanol; rather, loss of aversion learning after LHb lesion is the more likely cause of increased voluntary intake in these rats (Haack et al., 2014).

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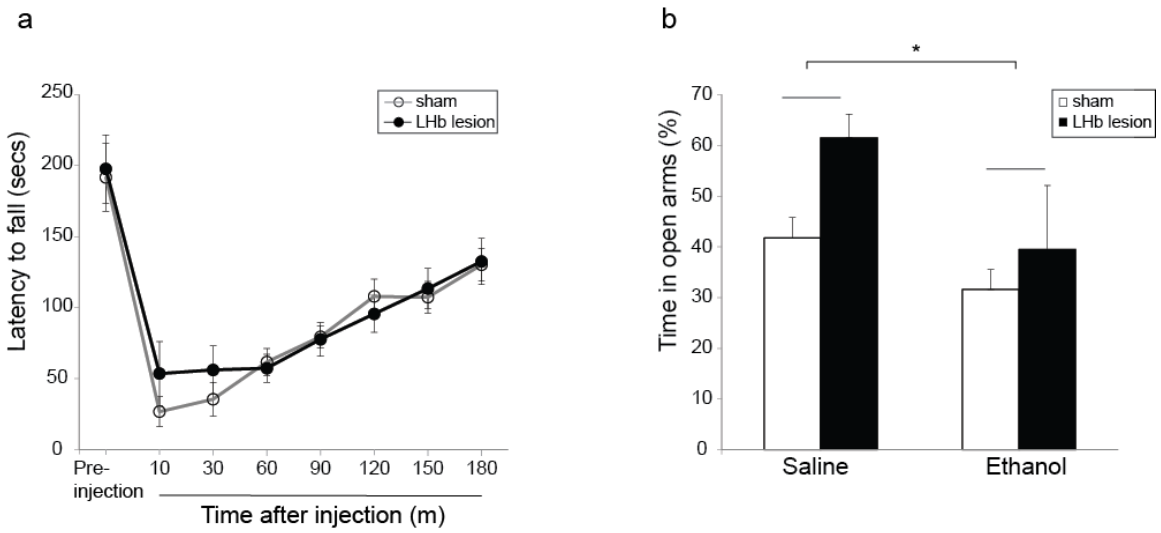

Figure 3.1 Rotarod and elevated-plus maze (EPM) (a) LHb lesion did not alter ethanol-induced motor impairment on the rotarod. Data from LHb-lesioned rats are shown as closed circles and data from sham-lesioned rats are shown as open circles. Symbols depict mean latency to fall from the rotarod ± SEM. Time (x-axis) is shown in minutes (b) LHb lesion did not significantly affect ethanol-withdrawal-induced anxiety on the EPM test. However, LHb-lesioned rats show increased basal exploration of open arms. Data for sham-lesioned rats are shown as open bars while data for LHb-lesioned rats are shown as closed bars. Asterisk indicates main effect of drug.

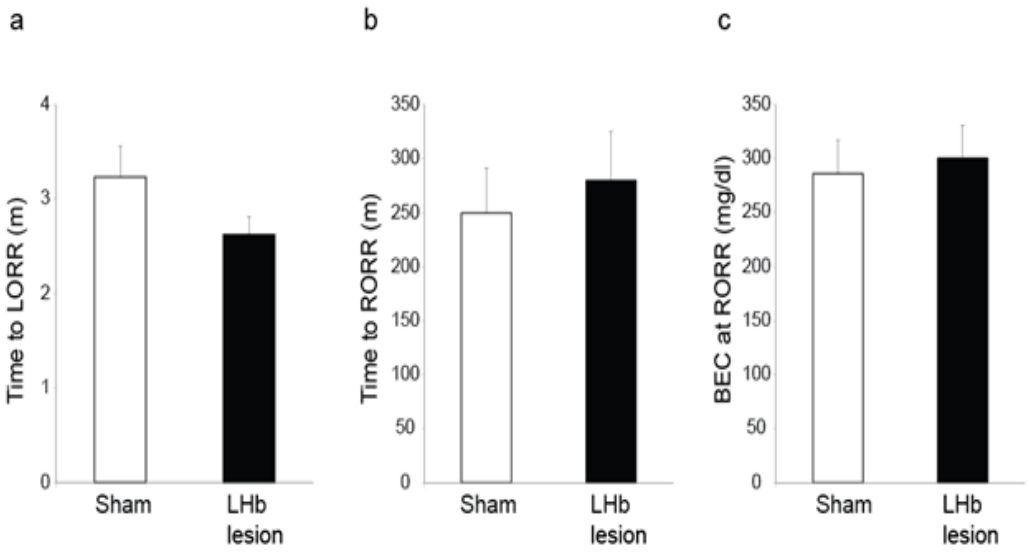

Figure 3.2 Loss of Righting reflex (a) LHb lesions did not alter the time required to lose righting reflex (LORR), or (b) the time required to regain the righting reflex (RORR). Time (y-axis) is shown in minutes. (c) Mean BEC at RORR is similar for LHb- and sham-lesioned rats. Sham-lesioned rats are shown as open bars and LHb-lesioned rats are shown as closed bars.

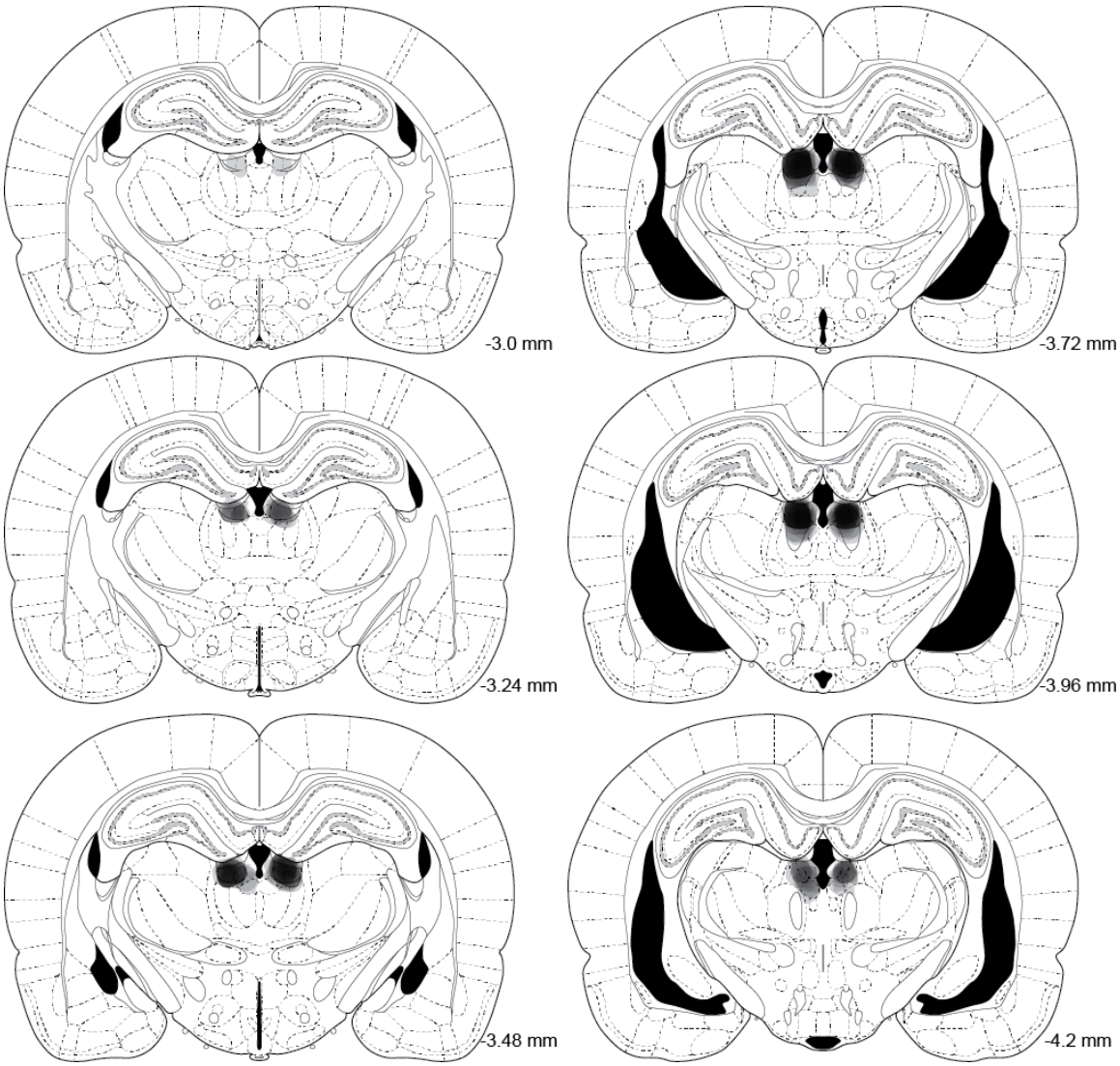

Figure 3.3 LHb lesion placements. Electrolytic lesion sites for each rat are overlaid such that the areas with the darkest shading have the maximum damage. Lesion sites were centered on LHb, with some damage to the medial habenula (MHb). Anterior-posterior coordinates relative to bregma are shown on the right of each coronal section.

CHAPTER 4

LESIONS OF THE ROSTROMEDIAL TEGMENTAL NUCLEUS INCREASE VOLUNTARY ETHANOL CONSUMPTION AND ACCELERATE EXTINCTION OF ETHANOL-INDUCED CONDITIONED TASTE AVERSION

Abstract

Ethanol has rewarding and aversive properties, and the balance of these properties influences voluntary ethanol consumption. Preclinical and clinical evidence show that aversive properties of ethanol limit intake. The neurological substrates mediating aversive properties of ethanol are not fully understood. I have previously shown that the LHb, a region critical for aversive conditioning, plays a role in ethanol-directed behaviors. However, the neural circuitry through which LHb exerts its actions is unknown. I investigated a role for the RMTg, a major LHb projection target, in regulating ethanol-associated behaviors. Rats received either sham or RMTg lesions and were studied during intermittent ethanol access (IEA), operant ethanol self-administration, extinction, yohimbine-induced reinstatement of ethanol-seeking, and ethanol-induced CTA. I found that RMTg lesions increased voluntary ethanol consumption in the IEA paradigm and

accelerated extinction of ethanol-induced CTA. These results lead me to the conclusion that the RMTg plays a crucial role in regulating voluntary ethanol consumption, likely by mediating ethanol-induced aversive conditioning.

Introduction

Drugs of abuse, including ethanol, have both rewarding and aversive properties (Verendeev and Riley, 2013), and the relative balance of these properties importantly influences levels of drug intake (Riley, 2011). The aversive effects of ethanol include nausea, motor impairment, sedation, and hangover-like effects (Schulteis and Liu, 2006). In humans, ethanol produces higher subjective rewarding and lower sedative responses in heavy vs. light drinkers, suggesting that increased reward, as well as decreased aversion, in response to ethanol consumption can contribute to heavy ethanol intake (King et al., 2011). Further, for heavy drinkers, higher subjective rewarding responses and lower aversive response are predictive of increased ethanol-binge frequency, as well as higher levels of alcohol-use disorder. Hence, resistance to the aversive effects of ethanol may increase vulnerability to ethanol abuse (King et al., 2011; King et al., 2014), highlighting the role of the aversive effects of ethanol in regulating its intake.

In rodent models, multiple lines of evidence suggest that the aversive effects of ethanol act to limit ethanol intake. For example, voluntary ethanol consumption in mouse strains is inversely correlated with ethanol-induced CTA (Green and Grahame, 2008), suggesting that mice that experience more

aversion to ethanol voluntarily consume less ethanol. In addition, adolescent rats voluntarily consume more ethanol and are less sensitive to the aversive effects of ethanol compared to adult rats (Vetter-O'Hagen et al., 2009). Further, alcohol-preferring rats (P) rats do not decrease voluntary intake of ethanol after involuntary ethanol pre-exposure, in stark contrast to wild-type Sprague-Dawley rats (Rezvani et al., 2010). Lower sensitivity to the aversive effects of ethanol could be one of the potential contributors to high ethanol intake in P rats. Thus, both preclinical and clinical studies provide strong evidence that attenuated aversion to ethanol is associated with high levels of voluntary ethanol consumption.

Understanding the neural circuits underlying ethanol's aversive effects is therefore likely to be important in understanding the neural mechanisms underlying escalation of ethanol intake and vulnerability to addiction. The lateral habenula (LHb) is an epithalamic brain region that importantly regulates aversive conditioning and has been implicated in regulating voluntary ethanol intake (Matsumoto and Hikosaka, 2007, 2009a; Haack et al., 2014). The role of the LHb in such processes is thought to be mediated, in part, through inhibition of midbrain DA neurons (Christoph et al., 1986; Ji and Shepard, 2007) that encode reward prediction errors and regulate reinforcement learning (Schultz, 1998). This inhibition is mediated through a disynaptic relay, with the midbrain rostromedial tegmental nucleus (RMTg) serving as the relay structure (Jhou et al., 2009b; Hong et al., 2011). The LHb sends glutamatergic efferents to the principally GABAergic neurons of the RMTg, which in turn inhibit VTA DA

neurons (Jhou et al., 2009b). Like LHb stimulation, *in vivo* stimulation of the RMTg inhibits midbrain DA neurons (Lecca et al., 2012), suggesting that the LHb-RMTg pathway acts as a 'brake-pedal' on midbrain DA neuron firing.

Given that the VTA DA neurons provide a "teaching signal" that drives reward learning and subsequent approach or appetitive behaviors (Schultz, 2007a), it is possible that the LHb-RMTg circuit provides an opposite "aversive teaching signal", which drives aversive learning and subsequent avoidance behaviors. Indeed, optogenetic stimulation of LHb terminals in RMTg causes active, passive, and conditioned avoidance (Stamatakis and Stuber, 2012), and both conditioned and unconditioned aversive stimuli increase c-*fos* induction in RMTg neurons that receive afferent input from the LHb and project to the VTA (Jhou et al., 2009a). Finally and importantly, cocaine-induced conditioned avoidance is abolished by lesioning either LHb efferents (the FR) or the RMTg (Jhou et al., 2013). These results provide evidence that the LHb-RMTg circuit mediates learning driven by the aversive effects of cocaine, and ablating this pathway eliminates learned avoidance responses to cocaine (Jhou et al., 2013).

Given the robust efferent projection from the LHb to the RMTg, and the role of the RMTg in avoidance behaviors, I hypothesized that the RMTg also plays an important role in regulating ethanol-directed behaviors. In the present study, I studied voluntary ethanol consumption, operant responding, yohimbine-induced reinstatement, and ethanol-induced CTA in RMTg- and sham-lesioned rats.

I provide evidence of a role for the RMTg in regulation of voluntary ethanol

intake and in extinction of ethanol-induced CTA. These results, combined with previous findings (Haack et al., 2014), suggest that the LHb and RMTg play tightly coupled roles in regulating voluntary ethanol intake, likely by mediating ethanol-induced aversive conditioning.

Materials and methods

Subjects

Forty-two male Long-Evans rats were used (300-350 g on receipt; Charles-River, Wilmington, MA). Rats were single-housed in Plexiglas tub cages and maintained on a 12 hour (h) light/dark cycle. *Ad libitum* access to food and water was available at all times except during conditioned taste aversion experiments (see below for details). All procedures occurred in the light cycle (12:12 h), with lights on at 6 AM unless otherwise stated. All procedures used were approved by the University of Utah Animal Care and Use Committee and carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* (8^{th} edition).

Drugs

Ethanol solutions (Decon Labs, King of Prussia, PA) were prepared in filtered tap water to a concentration of 20% (v/v) for use in the IEA paradigm and as a 20% v/v solution in physiological saline for the CTA experiment. All other drugs were purchased from Sigma Aldrich (St. Louis, MO). Saccharin and

quinine solutions were prepared in distilled water. Yohimbine was prepared at a concentration of 4mg/ml in distilled water.

RMTg lesions

Bilateral excitotoxic lesions of the RMTg were produced using quinolinic acid (0.4 µl of 0.12 M solution in each hemisphere). Infusions were made via a 31 gauge needle connected to polyethylene tubing (PE50) attached to a 1 µl glass Hamilton (Reno, NV) syringe on a Harvard 2000 microinfusion pump (Harvard Apparatus, Holliston, MA). For each infusion, a volume of 0.4 µl was injected at a rate of 0.2 µl/min, with the needle left in place for an additional 2 minutes to allow for diffusion. RMTg coordinates were AP:-7.1 mm, ML: ± 1.2 mm, and DV: - 8.0 mm, relative to bregma (Paxinos and Watson, 2007b). For sham lesions, the needle was lowered 1 mm dorsal to the RMTg, but no infusion was made.

Intermittent ethanol access (IEA)

Voluntary ethanol consumption was monitored for 8 weeks using a two-bottle choice IEA paradigm in a total of 42 rats (21 sham- and 21 RMTg-lesioned). However, 1 RMTg-lesioned rat died during IEA, data from 2 RMTg-lesioned rats were eliminated due to misplaced lesions, and 1 RMTg-lesioned rat that never consumed ethanol during the IEA was also eliminated from analysis, resulting in a final sample size of 21 sham- and 17 RMTg-lesioned rats.

In the IEA paradigm, rats were given 24-hour access to two bottles in their home cages on alternate weekdays. One bottle contained 20% ethanol (v/v) in

tap water and the other contained tap water. On Monday, Wednesday and Friday of each week, the bottles were weighed and placed in the home cages at 9 AM, and then removed and weighed at 9 AM the following day so that total consumption was recorded for each 24-h session. The position of each bottle was alternated on successive sessions to minimize side preferences. *Ad libitum* water was available at all times. Food was available *ad libitum* at all times and food intake and body weight were measured weekly for all rats. In analyzing ethanol intake, total ethanol intake was normalized to body weight (g/kg/24 h), and ethanol preference was calculated [ethanol intake/total fluid (water +ethanol) intake].

Taste preference: two-bottle choice for saccharin and quinine solution

Taste preference and taste aversion were assessed using two-bottle choice paradigms comparing the intake of water to that of saccharin and quinine solutions, respectively, in a subset of rats (6 sham- and 6 RMTg-lesioned). Quinine intake was measured first (6 sessions) followed by saccharin intake (6 sessions). Each session consisted of a 48-h period in which two bottles were provided in the home cage. One bottle contained distilled water, and the second bottle contained either quinine or a saccharin solution. Tastant concentrations increased across sessions [0.001, 0.003, 0.01, 0.03, 0.1, and 0.3 mM concentrations for successive quinine sessions; 0.01, 0.05, 0.1, 0.5, 1, and 5 mM concentrations for successive saccharin sessions]. Consumption was recorded at 24-h intervals, and the side of the bottle was switched at this time to minimize

side preferences. Quinine and saccharin preference for each concentration (i.e., each 48-h period) was calculated by averaging the intake for the two 24-h periods and then dividing by the average total fluid intake.

Measurement of blood ethanol concentration (BEC)

In 12 rats (6 sham- and 6 RMTg-lesioned) that had received 8 weeks of IEA, BECs were measured after voluntary ethanol intake during the IEA. Specifically, tail vein blood was collected after the first 30 minutes of ethanol access, the interval during which previous results suggest intake rates are highest (Carnicella et al., 2009; Haack et al., 2014). BEC measurements were performed as described in Chapter 3.

Operant responding for ethanol

Operant responding for ethanol was investigated in a subset of rats (15 sham- and 14 RMTg-lesioned) that had first undergone the IEA procedure. However 5 sham- and 3 RMTg-lesioned rats were eliminated from analysis since they failed to reach a threshold criterion of 0.3g/kg/h of ethanol intake (Simms et al., 2010; Bertholomey et al., 2013). In addition, 2 RMTg-lesioned rats were excluded due to improper lesion placement, leading to inclusion of data from 10 sham- and 9 RMTg-lesioned rats in the final analysis. Training occurred in 8 Med Associates chambers (St. Albans, VT), enclosed in sound-attenuating cabinets and equipped with ventilation fans. Each chamber contained a recessed magazine where 20% ethanol could be delivered via a programmable syringe

pump. The magazine was flanked by two retractable levers, and illuminated cue lights were positioned above each lever. The right lever always served as the active lever. Responding on the active lever extinguished the cue light, retracted the lever, and delivered 0.1 ml ethanol into the magazine. After a 5-second time-out period, the lever was extended and the cue light again illuminated. The lever located to the left of the magazine served as the inactive lever. Responding on the inactive lever had no programmed consequences. In early training sessions, only the active lever was present and every lever response was reinforced (i.e., FR1 schedule). After an initial overnight session, rats were trained daily in 1-h sessions until they responded at stable levels (less than 20% variability between 2 sessions). Rats reached this criterion after 3.9±0.4 (sham-lesioned) and 3.8±0.4 sessions (RMTg-lesioned, no significant difference, $p=0.58$). The response requirement was then increased to an FR3 schedule (i.e., every third lever press was reinforced). Rats were trained on this paradigm for 2 sessions, after which the inactive lever was introduced as a measure of nonspecific responding. All rats were trained on this final paradigm for 7 sessions. The responses on the active lever were averaged across the last 3 sessions for each rat as a final measure of operant responding.

Extinction and reinstatement of ethanol-seeking

Next, extinction and reinstatement of operant responding were tested in the same group of rats (10 sham- and 9 RMTg-lesioned). Extinction sessions were identical to operant training sessions except that the syringe containing

ethanol was removed from the syringe pump. Thus, responding on the active lever resulted in retraction of the lever, extinguishing of the cue light, and activation of the syringe pump, but no ethanol delivery. Extinction sessions were conducted daily. Once extinction responding declined to 15 or fewer active lever presses per session for 3 consecutive sessions, rats were tested for yohimbine-induced reinstatement. Yohimbine (2 mg/kg, IP) or vehicle solution (distilled water) was administered 30 minutes prior to testing responses in a 90-minute extinction session. A longer extinction session (vs. 60 minute training sessions) was used to ensure reliable and robust yohimbine-induced reinstatement in control rats (Gill et al., 2013). Each animal received two injections of yohimbine and two injections of the vehicle solution, with the injection schedule counter-balanced across rats. Each test session that included an injection was separated by an extinction session without injection to ensure reinstated responding was reduced to criterion rates of extinction responding. Responses were then averaged for the two tests for each rat.

Ethanol-induced conditioned taste aversion (CTA)

A total of 29 rats (15 sham- and 14 RMTg-lesioned) were subjected to a CTA paradigm based on a previously published protocol (Rinker et al., 2011). Rats were first water deprived for 24 hours. They then received 20 minutes daily access to tap water in their home cage for 3 successive sessions. Rats were then given access to saccharin (0.125% in tap water) for 20 minutes in their home cages and consumption measured. Rats were immediately divided into 4

groups matched for saccharin consumption: sham-vehicle, sham-ethanol, RMTg lesioned-vehicle and RMTg lesioned-ethanol. Rats were then injected with 1.5 g/kg body weight of 20% v/v ethanol (IP) or an equivalent volume of vehicle (saline). On each of the following 2 days, rats received 20 minutes access to tap water in their home cages. This 3-day cycle (saccharin access followed by injection, followed by two days of water access without injection) was repeated a total of 3 times. CTA was then extinguished through administration of additional 3-day cycles in which there was no injection following each period of saccharin access. These extinction trials continued until saccharin consumption returned to preinjection baseline levels. Trials 1-3 were the conditioning trials in which saccharin access was paired with ethanol injection, while trials 4-14 were extinction trials in which saccharin access was not paired with ethanol. During trials 4 and 5 (the first two extinction trials), we noted marked variability in rats' latency to initiate lick after given access to saccharin. Thus, starting with the sixth trial, the latency to initiate lick was measured with a timer by an observer who was blind to the treatment condition.

Verification of lesions

Brain sections were immunostained for neuronal nuclei (NeuN) (Furlong and Carrive, 2007). Briefly, sections were prepared in 50% ethanol, 3% H2O2, and 5% normal horse serum, and then incubated in Mouse-anti-NeuN (1:5000 for 24-h; Merck-Millipore), followed by anti-mouse IgG (1:1000 for 24-h; Vector Laboratories) and Vectastain ABC reagent (1:750 for 2-h; Vector Laboratories).

Finally, a black reaction product was created using a nickel-intensified diaminobenzidine (DAB) reaction. Sections were then mounted onto gelatin-coated slides, dried, cleared in xylene, and cover slipped with DPX mounting medium. Lesions were verified using a light microscope, and plotted on templates modified from a reference rat brain atlas (Paxinos and Watson, 2007b).

Though damage was largely confined to RMTg, some lesion sites encroached upon nearby structures, including the interpeduncular nucleus (IPN), median raphe (MRN), and posterior VTA (pVTA). To determine if damage to each of these structures contributed to voluntary ethanol intake in the IEA paradigm, we quantified the damage to IPN, MRN, and pVTA in each RMTg-lesioned rat. The damage to each of these structures was assessed by visual inspection and was scored as 0% (no damage), 25%, 50%, 75%, and 100 % (complete ablation) in each hemisphere. Scores for each hemisphere were averaged to produce a single estimate of damage to the IPN, MRN, and pVTA in each RMTg-lesioned rat. Rats were divided into high- and low-damage groups by performing a median split analysis, and ethanol consumption in IEA paradigm was compared between the two groups.

Statistical analyses

Lesion effects on BEC after IEA, operant ethanol self-administration, and ethanol intake during 1st and 8th week of IEA were analyzed using t-tests. Voluntary ethanol consumption, ethanol preference, water intake, and total fluid

intake during IEA were analyzed using two-way RM ANOVA (factors of lesion and drinking session). Escalation of ethanol intake, taste preference, extinction of ethanol-seeking, and yohimbine-induced reinstatement were also analyzed using two-way RM ANOVA (factors of lesion and time, tastant concentration, extinction session, or drug, respectively). CTA results were analyzed using three-way RM ANOVA (factors of lesion, drug, and trial). JMP Pro 11 (SAS Institute Inc., Cary, NC) was the statistical software used to carry out analyses. Analyses were considered significant when $p<0.05$. Data is shown as mean ± S.E.M.

Results

Voluntary ethanol consumption

Intermittent access to ethanol increased ethanol intake in both sham- and RMTg-lesioned animals over the study period of 8 weeks (Figure 4.1a; significant main effect of drinking session, $F(3.7,133.1)=16.4$, $p<0.0001$). However, RMTg-lesioned rats escalated their ethanol intake faster and plateaued at higher ethanol intake levels at the end of 8 weeks (significant main effect of lesion, $F(1,36)= 4.7$, $p<0.05$ and significant interaction of lesion and drinking session, $F(3.7,133.1)=3.6$, $p<0.01$). Posthoc testing revealed that RMTg-lesioned rats drank more ethanol from session 13 through session 24 as compared to sham-lesioned animals ($p<0.05$). RMTg-lesioned rats also had higher preference for ethanol relative to sham-lesioned rats (Figure 4.1b; significant main effect of lesion, $F(1,36)=4.2$, $p<0.05$, and significant main effect of drinking session,

F(5.5,196.8)=21.8, p<0.0001,no significant interaction of lesion and drinking session, F(5.5,196.8)=1.6, p=0.16).

Water intake declined progressively for both sham- and RMTg-lesioned groups during the 8 weeks of IEA (Figure 4.1c; significant main effect of drinking session, F(4.9,177.1)=14.3, p<0.0001). RMTg lesion had no significant effect on water intake (no significant main effect of lesion, F(1,36)=0.3, p=0.6, and no significant interaction of lesion and drinking session, F(4.9,177.1)=0.9, p=0.47). Further, total fluid intake did not differ between sham- and RMTg-lesioned rats (Figure 4.1d; no significant main effect of lesion, F(1,36)=2.1, p=0.16 and no significant interaction of drinking session and lesion, F(3.8,137.9)=1.2, p=0.33). Also, RMTg lesion did not alter weekly food intake (150.6± 4.1g/week and 158.1±7.7g/week for sham- and RMTg-lesioned rats respectively; no significant main effect of lesion, F(1,25)=0.6, p=0.46, and no significant interaction of lesion and time, F(4.5,112.5)=2, p=0.09).

BEC analyzed from tail vein blood obtained after the first 30 minutes of access to ethanol in the IEA paradigm revealed a significant correlation between BEC and ethanol intake normalized to weight (Figure 4.1e; r2=0.72 and r2=0.71 for sham- and RMTg-lesioned respectively; p<0.05 for each group, Pearson's correlation). However, the difference in BEC did not reach significance in comparing RMTg- vs. sham-lesioned rats, although there was a strong trend towards significance (Figure 4.1f; t=1.6, p=0.068).

I analyzed differences in ethanol intake between sham- and RMTg-lesioned rats at the start (week 1) and end (week 8) of the IEA paradigm. Sham-

and RMTg-lesioned groups did not differ significantly in their average ethanol intake during week 1 (Figure 4.2a; $t=-0.8$, $p=0.21$). However, RMTg-lesioned rats had significantly higher ethanol intake during week 8 as compared to sham-lesioned animals (Figure 4.2b; $t=-2.9$, $p<0.005$). The higher ethanol intake observed in RMTg-lesioned rats during week 8 can be attributed to more rapid escalation of intake, particularly during the first 5 weeks of IEA. Analysis of the slope of ethanol intake during the first 5 weeks (sessions 1-15) vs. last 3 weeks (sessions 16-24) showed that overall, the average slopes were significantly higher in the RMTg- vs. sham-lesioned rats, and the rate of escalation was significantly higher in the first 5 weeks as compared to the last 3 weeks (Figure 4.2c; significant main effect of lesion, $F(1,36)=4.6$, $p<0.05$ and significant main effect of time, $F(1,36)=4.4$, $p<0.05$).

Taste preference

Ethanol has both bitter and sweet taste components (Scinska et al., 2000; Blizard, 2007). To rule out the possibility that higher ethanol intake in RMTg-lesioned rats was due to changes in taste preference, I studied quinine (bitter) and saccharin (sweet) preference in sham- and RMTg-lesioned rats. Quinine aversion increased in both sham- and RMTg-lesioned groups with increasing quinine concentration (Figure 4.3a; significant main effect of quinine concentration, $F(3,29.5)=48.1$, $p<0.0001$). However, RMTg lesion had no significant effect on quinine aversion (no significant main effect of lesion, $F(1,10)=2$, $p=0.19$ and no significant interaction of lesion and quinine

concentration, $F(3,29.5)=2.1$, $p=0.12$). Saccharin preference increased with increasing saccharin concentration for both sham- and RMTg-lesioned rats (Figure 4.3b; significant main effect of saccharin concentration, $F(3.4,34.5)= 99.1$, $p<0.0001$). Again, RMTg lesion had no significant effect on saccharin preference (no significant main effect of lesion, $F(1,10)=1$, $p=0.33$ and no significant interaction of lesion and saccharin concentration, $F(3.4,34.5)=1$, $p=0.39$).

Operant self-administration of 20% ethanol

RMTg- and sham-lesioned rats did not differ in the average number of active lever presses across the last 3 sessions of operant training (Figure 4.4a; $t=-0.8$, $p=0.21$), nor were there differences in inactive lever presses between the two groups (data not shown, $t=0.9$, $p=0.17$). In addition, the level of ethanol intake across the last 3 sessions did not differ between the two groups (0.59 ± 0.05 vs. 0.66 ± 0.06 g/kg/24 h for sham and RMTg-lesioned rats respectively, $t=-0.95$, $p=0.17$). Sham and RMTg-lesioned rats progressed similarly through the seven FR3 sessions (no significant main effect of lesion, $F(1,17)=0.6$, $p=0.45$, no significant interaction of lesion and session, $F(4.6,78.2)=0.2$, $p=0.95$ but main effect of time, $F(4.6,78.2)=5.3$, $p<0.0005$).

Extinction and yohimbine-induced reinstatement of ethanol-seeking

Analysis of operant responding through the first 5 extinction sessions revealed that both sham- and RMTg-lesioned rats reduced operant responding

as the extinction sessions progressed (Figure 4.4b; significant main effect of extinction session, $F(2.6,44.9)=24.2$, $p<0.0001$). However, RMTg lesion had no significant effect on the rate of extinction (no significant main effect of lesion, $F(1,17)=2.5$, $p=0.13$ and no significant interaction of lesion and extinction session, $F(2.6,44.9)=1$, $p=0.39$). I also analyzed the average number of extinction sessions required to reach the extinction criterion. There was no statistically significant difference between sham- and RMTg-lesioned rats (Figure 4.4c; $t=-1$, $p=0.15$). Also, RMTg lesion had no significant effect on inactive lever presses during extinction (data not shown; no significant main effect of lesion, $F(1,17)=1.09$, $p=0.31$, and no significant interaction of lesion and extinction session, $F(5,85)=0.2$, $p=0.96$).

Yohimbine administration reinstated operant responding in both sham- and RMTg-lesioned rats (Figure 4.4d; significant main effect of drug, $F(1,17)=19.5$, $p<0.001$). However, RMTg lesion did not alter yohimbine-induced reinstatement of ethanol-seeking (no significant main effect of lesion, $F(1,17)=0.001$, $p=0.97$ and no significant interaction of lesion and drug $F(1,17)=0.1$, $p=0.75$). Neither yohimbine administration nor RMTg lesion had significant effects on inactive lever presses during reinstatement sessions (data not shown; no significant main effect of drug, $F(1,17)=2.7$, $p=0.12$; no significant main effect of lesion, $F(1,17)=1.9$, $p=0.18$; no significant interaction of lesion and drug, $F(1,17)=0.6$, $p=0.43$).

Ethanol-induced conditioned taste aversion

Ethanol (1.5g/kg) conditioned a robust conditioned taste aversion in both sham- and RMTg-lesioned rats (Figure 4.5a; significant main effect of drug, $F(1,22)=61.7$, $p<0.0001$; significant main effect of trial, $F(5.3,115.8)=39.1$, $p<0.0001$ and significant interaction of drug and trial, $F(5.3,115.8)=18.1$, $p<0.0001$), as indicated by a reduction in saccharin consumption for both groups after pairing with ethanol injection. However, extinction of CTA was dependent on lesion (Figure 4.5a; significant main effect of lesion, $F(1,22)=5.2$,$p<0.05$; significant interaction of lesion and trial , $F(5.3,115.8)=2.3$, $p<0.05$ and significant interaction of lesion and drug, $F(1,22)=5$, $p<0.05$), showing that RMTg-lesioned rats recovered from aversion significantly faster than sham-lesioned. Importantly, there was a significant 3-way interaction of lesion, drug, and trial ($F(5.3,115.8)=2.3$, $p<0.05$), suggesting that there were time-dependent differences in ethanol-induced CTA effects on sham- vs. RMTg-lesioned rats. Posthoc tests revealed that RMTg-lesioned rats injected with ethanol extinguished CTA more rapidly than sham-lesioned counterparts (significant posthoc differences on trial 4 and from trial 7 to 14, $p<0.05$). There were no posthoc differences between sham- and RMTg-lesioned rats injected with vehicle solution.

I also analyzed the latency to initiate lick during trials 6-14 (extinction trials). A two-way RM ANOVA revealed that RMTg-lesioned rats initiated saccharin consumption significantly earlier than sham-lesioned animals (Figure 4.5b; significant main effect of lesion, $F(1,11)=9.9$, $p<0.01$). Average latency to initiate lick across trials 6-14 was significantly shorter in RMTg-lesioned rats

(Figure 4.5b; t=3.4, p<0.05).

Histological confirmation of lesions for RMTg

Lesions were largely confined to the RMTg (Figure 4.6). Damage encroached upon neighboring structures including the IPN, MnR, and pVTA in a few cases. To determine if damage to these areas contributed to increased ethanol intake in RMTg-lesioned rats, I quantified the damage for each of the areas for each RMTg-lesioned rat tested in the IEA paradigm.

I found no significant differences between low- and high-damage groups in their ethanol intake in the final drinking session in the IEA paradigm (Table 4.1, for IPN: average for low- and high-damage groups was 6.3±1 and 5.5±1.2 g/kg/24 h respectively, p=0.8, for MRN: average for low- and high-damage groups was 6.16 ± 1.1 and 6.14±1.4 g/kg/24 h respectively, p=0.5; for pVTA: average for low- and high-damage groups was 6.8±1.1 and 5.5±1.2 g/kg/24 h respectively, p=0.46, Student's t-test). In addition, I also analyzed if drinking levels throughout the IEA were different in the low- and high-damage groups using 2-way RM ANOVA. I did not find significant differences between low- and high-damage groups with respect to their ethanol intake throughout the IEA (For IPN: no significant main effect of group, F(1,15)=0, NS and no significant interaction of group and time, F(3.8,57)=0.35, NS, for MRN: no significant main effect of group, F(1,15)=0.04, NS and no significant interaction of group and time, F(3.9, 58.8)=0.8, NS and for pVTA: no significant main effect of group, F(1,15)=0.14, NS and no significant interaction of group and time, F(4.2,

62.8)=1.2, NS). I also wanted to rule out that damage to these areas contributed to accelerated extinction of ethanol-induced CTA. However, 5 out of 6 RMTg-lesioned rats injected with ethanol in CTA had very low damage to the surrounding areas (<median). Collectively, these data suggest that damage to the neighboring areas of the RMTg does not contribute to effects of RMTg lesion on voluntary ethanol consumption and ethanol-induced CTA.

Discussion

I studied the effects of RMTg lesion on voluntary ethanol consumption, operant ethanol self-administration, yohimbine-induced reinstatement, and ethanol-induced CTA in the current study. My findings indicate that this nucleus plays an important role in regulating voluntary ethanol intake, possibly by attenuating the strength of ethanol-induced aversive conditioning. In support of this conclusion, I show that RMTg-lesioned rats voluntarily consumed more ethanol in the IEA paradigm as compared to shams. Importantly, RMTg-lesioned rats also extinguished ethanol-induced CTA faster than sham-lesioned rats. RMTg lesions did not significantly change operant ethanol self-administration, extinction of ethanol-seeking or yohimbine-induced reinstatement of ethanol-seeking. I discuss the implications of these findings in more detail below.

Effects of RMTg lesion on voluntary ethanol consumption and ethanol-induced CTA

RMTg-lesioned rats showed increased ethanol consumption and ethanol preference in the IEA paradigm (Figures 4.1a and 4.1b) as compared to sham-lesioned rats. However, RMTg lesion did not acutely increase voluntary ethanol consumption, since these animals did not differ from sham-lesioned rats in ethanol consumption during the first week of IEA (Figure 4.2a). In contrast, RMTg-lesioned rats had significantly higher ethanol intake during the 8th week of IEA (Figure 4.2b). My results show that RMTg-lesioned rats escalated their ethanol intake faster during IEA (Figure 4.2c), suggesting that RMTg lesion affects the rate at which rats escalate their ethanol consumption during IEA rather than causing an acute increase in ethanol consumption. It is not likely that taste differences influenced ethanol consumption, given that there were no statistically significant differences in quinine aversion and saccharin preference between RMTg- and sham-lesioned rats (Figures 4.3a and 4.3b).

The increase in ethanol consumption in RMTg-lesioned rats may arise due to accelerated extinction of ethanol-induced CTA. My CTA results showed that ethanol injection conditioned a robust aversion to saccharin in both sham- and RMTg-lesioned groups. There was no difference between the groups in the magnitude of this aversion. However, RMTg lesions accelerated the rate at which rats extinguished CTA and returned to preinjection levels of saccharin consumption (Figure 4.5a). Interestingly, RMTg-lesioned rats initiated licking at the saccharin bottle faster than shams during extinction trials, again indicative of

faster CTA extinction (Figure 4.5b). My finding that RMTg lesion did not acutely increase voluntary ethanol consumption, but rather increased the rate at which voluntary intake escalated over time (Figure 4.3c), consistent with the notion that increased ethanol consumption in RMTg-lesioned rats resulted from an attenuation in the persistence of ethanol-induced aversive learning. Further experiments are needed to directly test if the increased ethanol consumption in RMTg-lesioned rats is a learned behavior caused by acceleration of extinction of ethanol-induced CTA. I note that some caution in interpreting our CTA results may be warranted. In my CTA study, I used a relatively high ethanol concentration (1.5g/kg) to induce a robust CTA in both groups. It is possible that floor effects could have masked differences in CTA between the groups. Possibly, a lower dose of ethanol might have revealed an attenuation of CTA in RMTg-lesioned rats.

Neural circuits underlying escalation of ethanol intake

My current results are consistent with and extend previous findings in which I showed that lesions of the LHb, which provides a major afferent input to RMTg, increase voluntary ethanol consumption and attenuate ethanol-induced CTA (Haack et al., 2014). The LHb inhibits VTA DA neurons through a disynaptic pathway involving the RMTg, in which the LHb sends a glutamatergic projection to the RMTg, which in turn sends a primarily GABAergic inhibitory projection to VTA DA neurons (Jhou et al., 2009b; Hong et al., 2011).

The LHb-RMTg-VTA pathway has been implicated in processing aversive

stimuli. The LHb shows increased neuronal activity as well as elevated c-*fos* expression in response to a range of aversive stimuli (Wirtshafter et al., 1994; Timofeeva and Richard, 2001; Matsumoto and Hikosaka, 2009a). Increased c-*fos* expression in response to both conditioned and unconditioned aversive stimuli is also seen in RMTg neurons (Hong et al., 2011; Brown and Shepard, 2013), specifically including those that receive afferents from LHb and project to VTA DA neurons (Jhou et al., 2009a). Finally, activation of LHb-RMTg projection is negatively reinforcing and produces active, passive and conditioned avoidance (Stamatakis and Stuber, 2012). These findings highlight the importance of the LHb-RMTg-VTA pathway in encoding aversion and avoidance behaviors.

Drug taking is governed by relative balance of drug-induced reward and aversion. The aversive effects of drugs of abuse serve as a limiting factor in regulating intake (Riley, 2011). Given the role of the LHb-RMTg projection in processing aversive stimuli and promoting avoidance behaviors, this pathway is well suited to play a central role in mediating drug-induced aversion learning that impacts voluntary drug intake. A substantial literature provides evidence that this anatomical pathway indeed plays this role. For instance, cocaine causes synaptic potentiation and increased excitability in LHb neurons that project to the RMTg (Maroteaux and Mameli, 2012). Increased firing of LHb neurons in turn causes negative depressive symptoms associated with cocaine withdrawal (Meye et al., 2015). Furthermore, cocaine causes delayed excitation in LHb neurons preferentially projecting to the RMTg, and that optogenetic inactivation of LHb terminals in the RMTg during this period of delayed excitation abolishes cocaine-

induced avoidance behavior (Jhou et al., 2013). In further agreement, a recent study reported that inhibiting the LHb abolishes ethanol-induced conditioned place aversion (CPA) (Zuo et al., 2015). Together, these results implicate activity in the LHb-RMTg pathway in driving aversive conditioning to cocaine and ethanol.

My current and previous (Haack et al., 2014) data add to the growing literature suggesting that the LHb-RMTg pathway can regulate drug intake, likely by mediating drug-induced aversive conditioning. Additional studies are needed to directly assess the effects of ethanol on RMTg neuronal firing and determine if ethanol-induced changes in neural firing cause ethanol-induced aversion.

Effects of RMTg lesion on operant ethanol self-administration, extinction, and yohimbine-induced reinstatement

A somewhat unexpected finding of the present work is that RMTg lesion did not cause significant changes in operant self-administration for ethanol (Figure 4.4a) or in yohimbine-induced reinstatement of ethanol-seeking (Figure 4.4d). I previously found that LHb lesion causes a significant increase in operant ethanol responding (Haack et al., 2014), and previous studies have found that LHb lesion blocks yohimbine-induced reinstatement of cocaine and ethanol-seeking (Gill et al., 2013; Haack et al., 2014). These findings suggest that the RMTg, unlike the LHb, does not play a major role in these processes. It is possible that LHb efferent targets other than the RMTg critically regulate these behaviors. The medial portion of LHb sends direct projections to the VTA

(Herkenham and Nauta, 1979; Goncalves et al., 2012) and to the serotonin (5-HT)-rich dorsal raphe (DR) and median raphe nuclei (MnR) (Herkenham and Nauta, 1979; Sego et al., 2014). In this later regard, yohimbine is an alpha-2 receptor antagonist which increases norepinephrine (NE) levels in the brain, leading to reinstatement of operant responding for most drugs of abuse (Shepard et al., 2004; Shalev et al., 2010). However, some evidence suggests that yohimbine's partial agonist activity at 5-HT1A receptors causes reinstatement (Dzung Le et al., 2009). In addition, various manipulations of the 5-HT-rich DR and MnR alter yohimbine-induced reinstatement (Le et al., 2013) and other stress-induced behavioral responses (Maier and Watkins, 2005; Dayan and Huys, 2009; Cools et al., 2011). Thus, it is possible that direct projections of the LHb to DR and/or MnR (Herkenham and Nauta, 1979; Sego et al., 2014), or some another projection target mediates LHb effects on yohimbine-induced reinstatement. Alternatively, with respect to operant responding, it is possible that our tests occurred too long after RMTg lesions were made, and that earlier training and testing in the operant paradigm might have revealed higher levels of intake by RMTg-lesioned rats.

RMTg lesion did not alter extinction of operant responding (Figures 4.4b and 4.4c). LHb lesions also had no effect on extinction of lever pressing (Haack et al., 2014). However, these findings contrast with a recent report showing that RMTg activity regulated extinction of cocaine-seeking (Huff and LaLumiere, 2014). Methodological differences (permanent excitotoxic lesions vs. acute infusion of AMPA potentiator) and the drug tested (ethanol vs. cocaine) could

account for the differences in findings.

Conclusions

The current findings, coupled with previous results (Haack et al., 2014) suggest that the LHb and the RMTg play important roles in regulating voluntary ethanol intake, likely by mediating aversive conditioning to ethanol. RMTg lesions, like lesions of the LHb, increased voluntary ethanol consumption and accelerated extinction of ethanol-induced CTA. Surprisingly, I found that the RMTg, unlike the LHb, does not play an apparent role in regulating ethanol-directed operant behavior and yohimbine-induced reinstatement of ethanol-seeking. These results provide evidence that the LHb-RMTg pathway regulates voluntary ethanol intake, and suggest pharmacologically targeted manipulations of this pathway may be promising in developing novel therapies for treatment of alcoholism.

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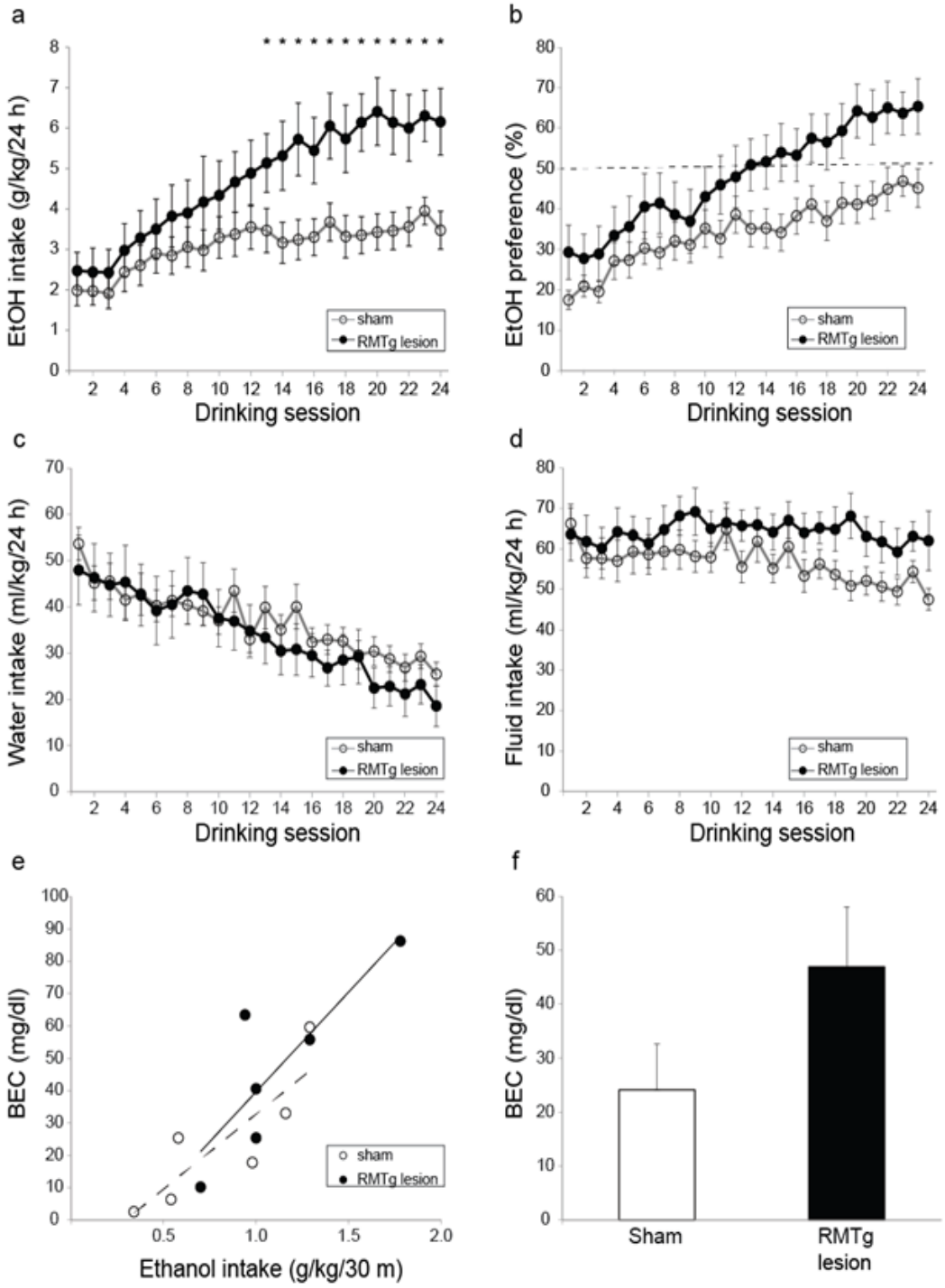
Figure 4.1 Effect of RMTg lesion on voluntary ethanol consumption (a) RMTg-lesioned rats voluntarily consumed significantly more 20% ethanol as compared to sham-lesioned animals. Open symbols represent data for sham-lesioned rats and closed circles represent data for RMTg-lesioned rats in this and all subsequent figures unless otherwise mentioned. Symbols depict mean ± SEM. Asterisks indicate significant posthoc differences between groups ($p<0.05$) (b) RMTg-lesioned rats had a higher preference for ethanol relative to sham-lesioned animals (c) Water intake decreased progressively in both groups; however, there were no significant differences between sham- and RMTg-lesioned rats (d) Total fluid intake did not differ between sham- and RMTg-lesioned rats (e) Blood ethanol concentration (BEC) was significantly correlated with ethanol intake in the first 30 minutes for both sham- and RMTg-lesioned rats. Broken line shows linear fit for sham-lesioned rats and solid line shows linear fit for RMTg-lesioned rats (f) BEC for RMTg-lesioned rats after voluntary intake was roughly twice as high as that measured for sham-lesioned animals.


a
EtOH intake (g/kg/24 h)
Drinking session
sham
RMTg lesion
b
EtOH preference (%)
Drinking session
sham
RMTg lesion
c
Water intake (ml/kg/24 h)
Drinking session
sham
RMTg lesion
d
Fluid intake (ml/kg/24 h)
Drinking session
sham
RMTg lesion
e
BEC (mg/dl)
Ethanol intake (g/kg/30 m)
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BEC (mg/dl)
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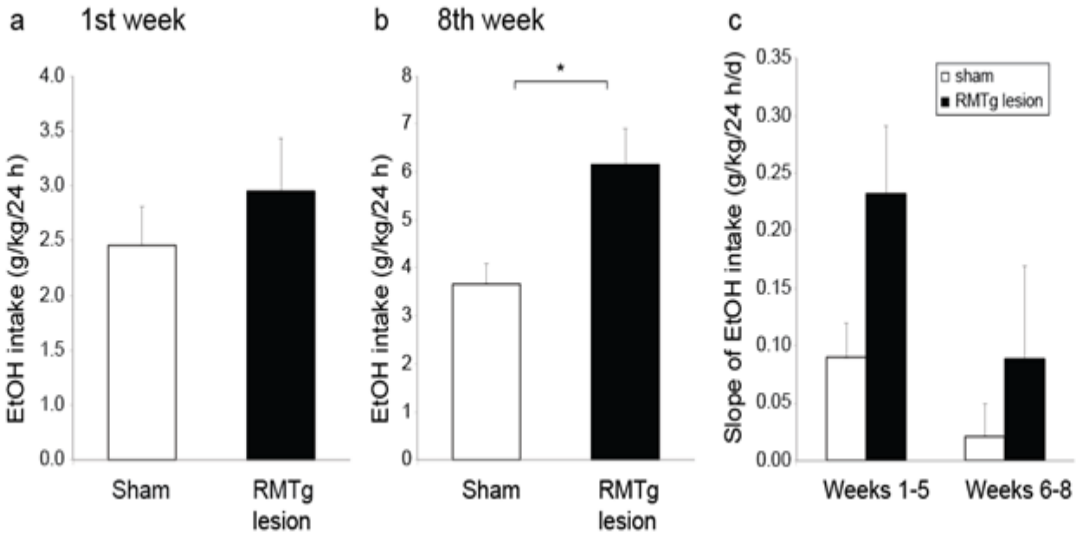

Figure 4.2 Effect of RMTg lesion on escalation of voluntary ethanol consumption (a) Mean ethanol consumption did not differ between sham- and RMTg-lesioned rats during the 1st week of IEA (b) Mean ethanol consumption in the 8th week was significantly higher in RMTg-lesioned rats (c) RMTg-lesioned rats escalated their ethanol intake faster as compared to shams.

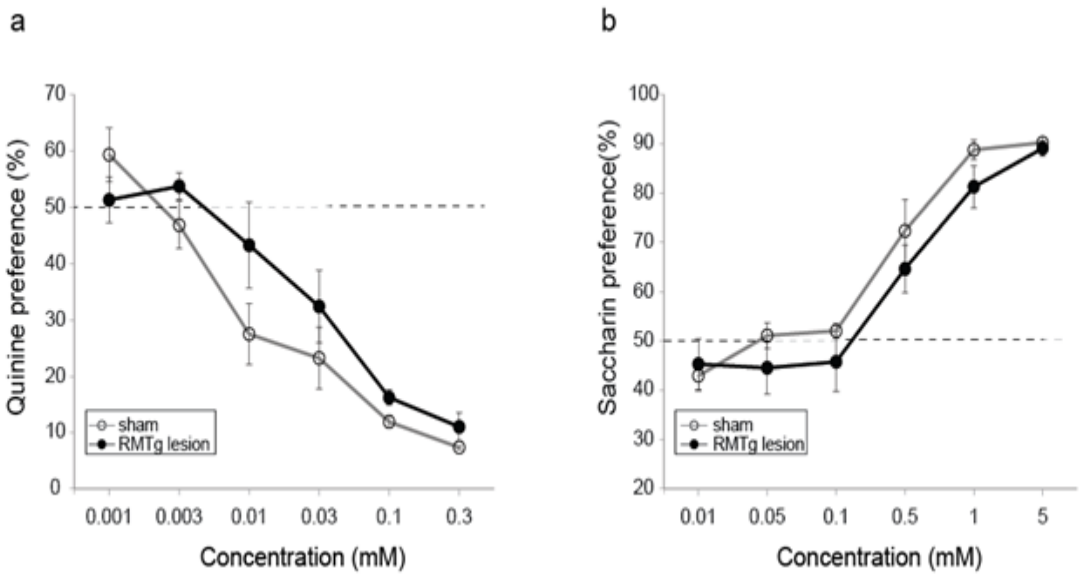

Figure 4.3 Effect of RMTg lesion on taste preference (a) RMTg lesion did not alter aversion to quinine or (b) preference for saccharin.

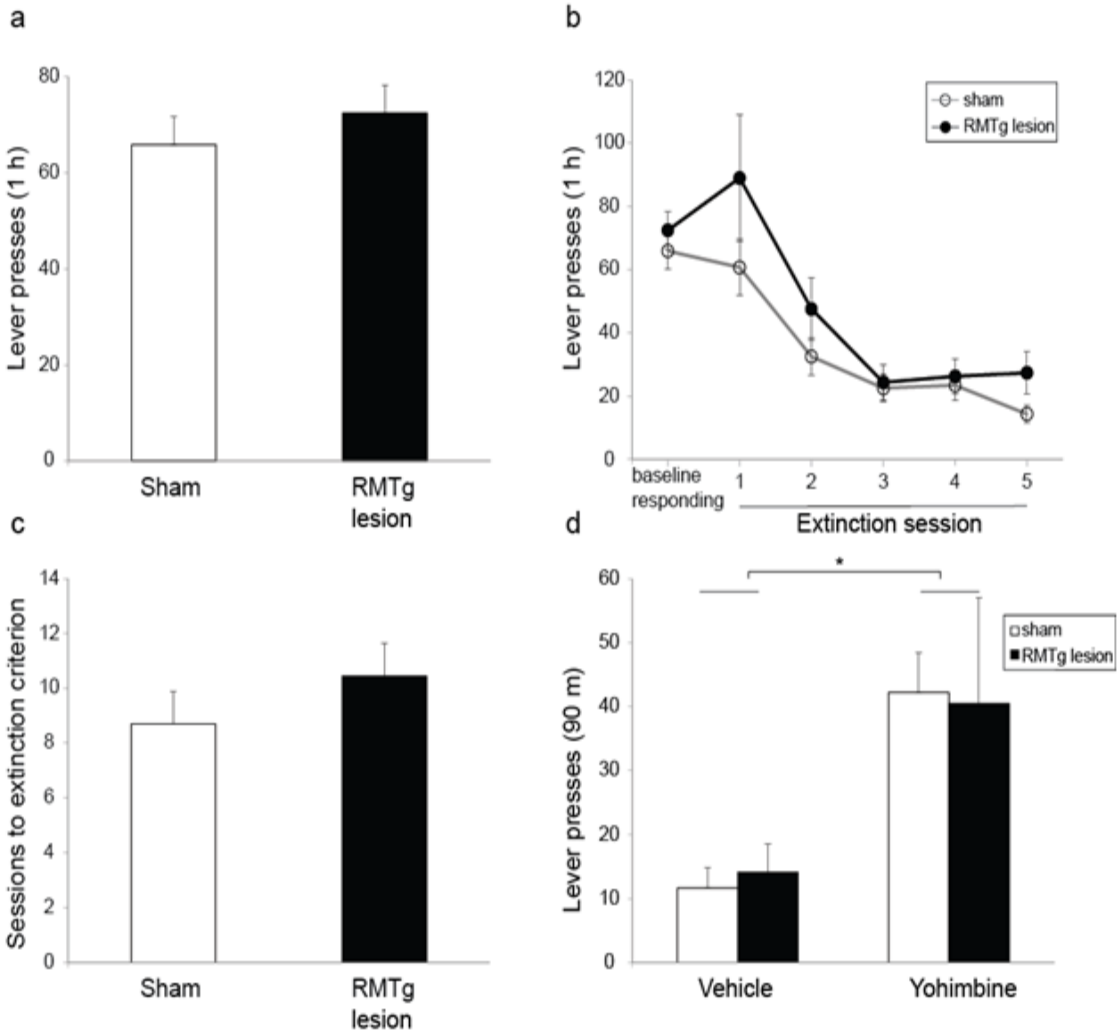

Figure 4.4 Effect of RMTg lesion on operant ethanol-seeking, extinction and yohimbine-induced reinstatement of ethanol-seeking (a) RMTg lesion did not significantly change operant responding for 20% ethanol or (b) the rate of operant extinction or (c) the mean number of extinction sessions required to reach the extinction criterion. (d) Yohimbine caused robust reinstatement in both sham- and RMTg-lesioned rats. Asterisk indicates main effect of drug.

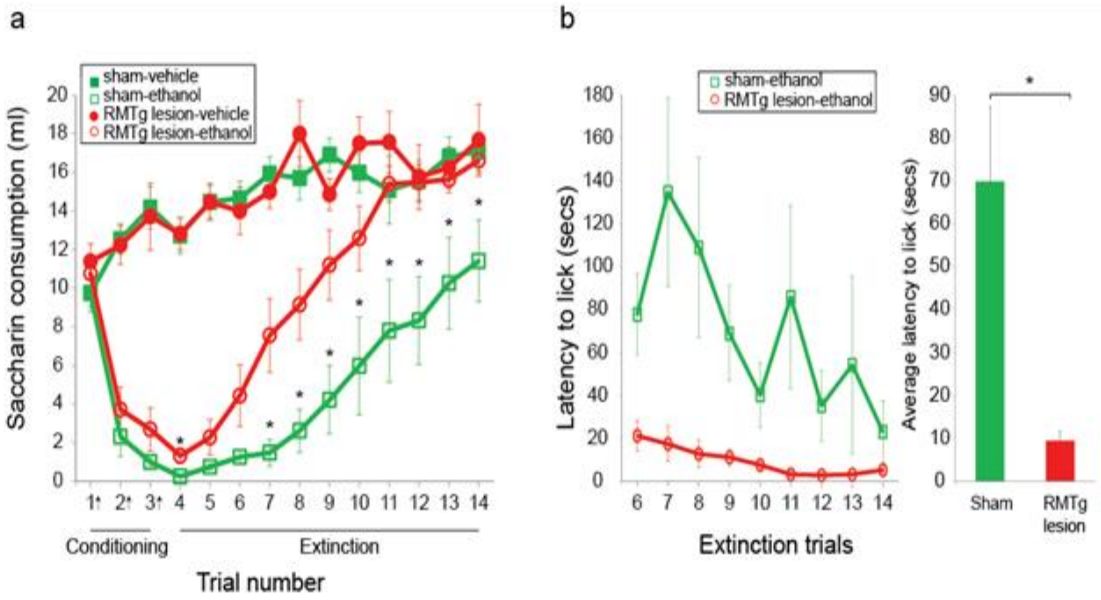

Figure 4.5 Effect of RMTg lesion on ethanol-induced CTA (a) RMTg lesion had no effect on the magnitude of an ethanol-induced CTA, but accelerated extinction of the CTA. Sham-lesioned rats are shown as squares and RMTg-lesioned rats are shown as circles. Open and closed symbols indicate treatment with ethanol and saline (vehicle), respectively. Arrows (x axis) indicate the trials in which saccharin access was paired with ethanol injection. Asterisks indicate significant differences between sham- and RMTg-lesioned groups who received ethanol injection ($p<0.05$) (b) RMTg lesion reduced the latency to initiate lick during extinction trials. Bar graph shows the average latency to initiate lick (seconds) for trials 6-14. RMTg-lesioned rats had significantly lower latency to initiate lick compared to sham-lesioned rats.

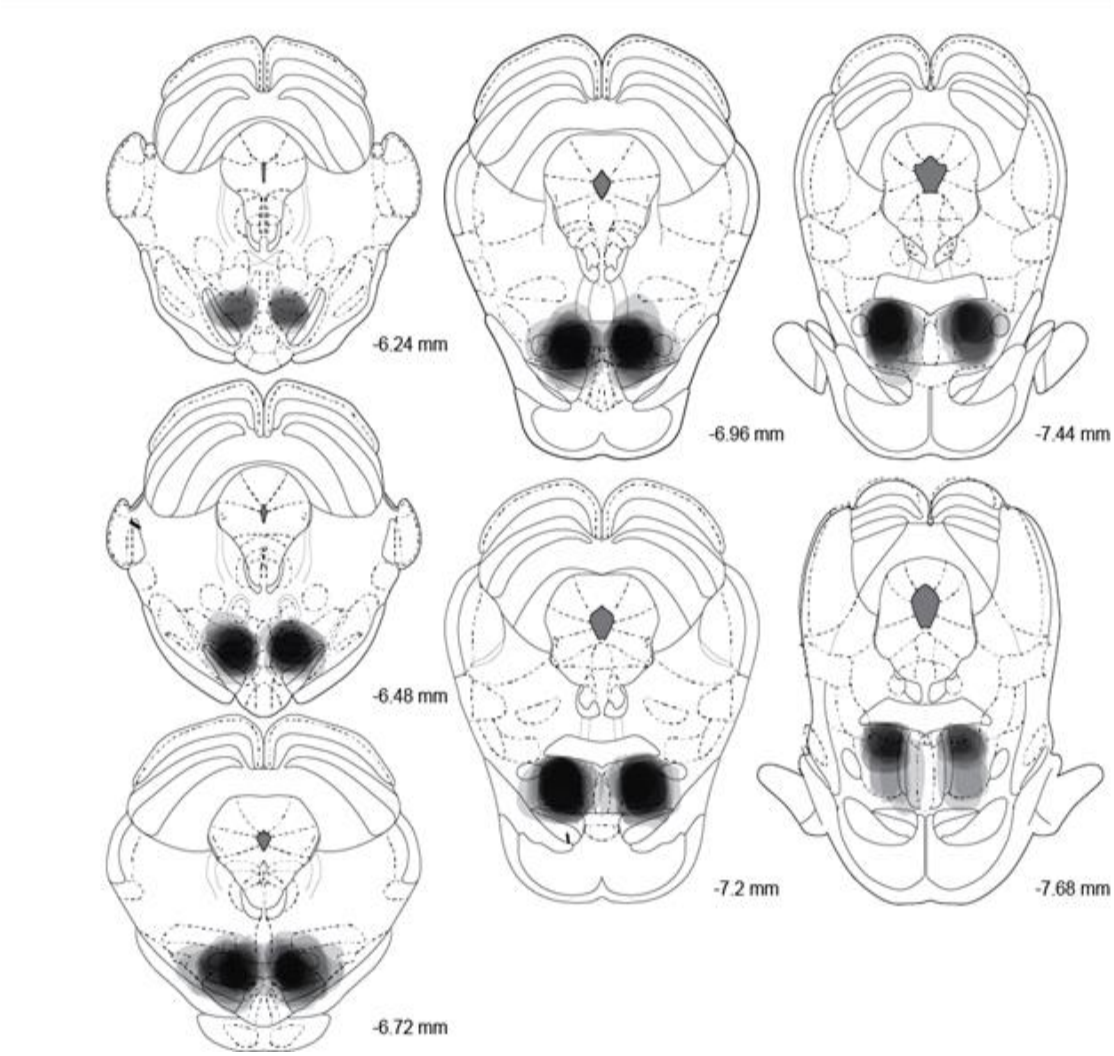

Figure 4.6 RMTg lesion placements. Excitotoxic lesion sites for each rat are overlaid such that the areas with the darkest shading have the maximum damage. Damage was restricted to RMTg in most cases; however, there was some damage to adjacent areas, including the median raphe and interpeduncular nucleus in some rats. The anterior-posterior coordinates relative to bregma are shown to the right of each coronal section.

Table 4.1 Quantification of ancillary damage to structures close to the RMTg in RMTg-lesioned rats. Rows indicate each brain structure analyzed. The first two columns indicate the volume of each brain structure lesioned in "small" and "large" lesion groups (determined by median split). Ethanol intake for each of the two groups is indicated for the small and large lesions groups in columns 3 and 4, respectively. The final column indicates significance values comparing levels of ethanol intake; there were no significant differences between any of the small and large lesions groups.

Brain structure	**Small lesion group** (% volume lesioned±SEM)	**Large lesion group** (% volume lesioned±SEM)	**Ethanol intake – small lesion group** (g/kg/24 h)	**Ethanol intake – large lesion group** (g/kg/24 h)	**p-value**
IPN	7±1	37±5	6.3±1.0	5.5±1.2	0.11
MNR	9±2	30±5	6.2±1.1	6.1±1.4	0.50
pVTA	23±2	39±2	6.8±1.1	5.5±1.2	0.46

CHAPTER 5

THE LATERAL HYPOTHALAMUS TO LATERAL HABENULA PROJECTION CONTROLS VOLUNTARY ETHANOL CONSUMPTION

Abstract

The LHb is an epithalamic brain locus implicated in aversive processing via negative modulation of midbrain DA and 5-HT systems. Given the role of the LHb in inhibiting DA and 5-HT systems, it has been postulated to be involved in various psychiatric pathologies, including drug addiction. In support, it has been shown that the LHb plays a critical role in cocaine and ethanol consumption behaviors, most likely be mediating drug-induced aversive conditioning. In previous work, I showed that lesions of the LHb increase voluntary ethanol consumption and operant ethanol self-administration, block yohimbine-induced reinstatement of ethanol-seeking, and attenuate ethanol-induced conditioned taste aversion. However, whether afferents to the LHb are required for mediating effects of the LHb on these behaviors remains to be investigated. Since my results showed that lesioning the fiber bundle which carries afferents to the LHb, the SM, increases voluntary ethanol consumption, I further investigated which

inputs to LHb mediate its effects on ethanol-directed behaviors. I specifically studied the role of the LH and VP inputs to LHb in ethanol-associated behaviors, given the involvement of the LH and VP in reward and motivation. The results show that the LH-LHb projection is necessary for regulating voluntary ethanol consumption, whereas, the VP-LHb projection is not necessary in mediating LHb effects on ethanol-directed behaviors. These results are an important first step towards understanding the functional role of afferent projections to the LHb with regard to drug-related behaviors.

Introduction

The LHb, an epithalamic structure in the brain, has been implicated in aversive processing (Hikosaka, 2010). For example, neurons within the LHb show increased and decreased activity in response to aversive and rewarding stimuli, respectively (Matsumoto and Hikosaka, 2007, 2009a). The LHb has an important role in regulating consumption of different drugs of abuse, including cocaine (Friedman et al., 2010) and ethanol (Haack et al., 2014), by mediating drug-induced aversive conditioning (Jhou et al., 2013; Haack et al., 2014; Zuo et al., 2015). Specifically, LHb lesions increase voluntary ethanol consumption in an IEA paradigm and reduce ethanol-induced CTA (Haack et al., 2014). LHb lesions also block yohimbine (stress)-induced reinstatement of cocaine- and ethanol-seeking, suggesting that the LHb modulates stress-induced drug-seeking (Gill et al., 2013; Haack et al., 2014). The LHb is thought to have its effects through negative modulation of midbrain DA and 5-HT neurons (Wang and Aghajanian,

1977; Christoph et al., 1986). Specifically, stimulation of the LHb inhibits activity of VTA DA neurons (Christoph et al., 1986) and 5-HT neurons in the raphe nuclei (Wang and Aghajanian, 1977) via a disynaptic pathway involving the RMTg (Jhou et al., 2009a; Hong et al., 2011). However, what afferents to the LHb are critical for mediating the effects of the LHb on ethanol-directed behaviors remains to be investigated.

The LHb receives afferent input from limbic forebrain, basal ganglia, and cortical structures through the SM (Herkenham and Nauta, 1977; Vadovicova, 2014). These structures include the LH and the VP (Herkenham and Nauta, 1977; Hong and Hikosaka, 2013; Poller et al., 2013), which are good candidates for mediating the effects of the LHb in ethanol-directed behaviors, given their role in appetitive and consummatory behaviors. For example, the LH is implicated in mediating reward-related behaviors (Aston-Jones et al., 2010; Millan et al., 2010) and also modulates ethanol consumption (Chen et al., 2013; Abulseoud et al., 2014; Chen et al., 2014). Specifically, administration of D1 agonists and D2 antagonists into the LH increases ethanol-intake, whereas administration of D1 antagonists and D2 agonists into LH reduces ethanol intake (Chen et al., 2014). Further, opioids in the LH suppress ethanol drinking (Chen et al., 2013). The VP is conceived of as the “final common pathway” for processing of reward and as a convergence point for hedonic and motivational signaling (Smith et al., 2009). It regulates ethanol consumption and ethanol seeking (Kemppainen et al., 2012; Perry and McNally, 2013; Khoo et al., 2015). Thus, both the LH and VP may serve as critical afferent regulators of the role of the LHb in ethanol-directed

behaviors.

The purpose of the present work, therefore, was to examine which afferents to the LHb are important for ethanol-directed behaviors. I first lesioned the SM to prevent all major inputs to LHb, and examined voluntary ethanol consumption using an IEA paradigm. I then specifically targeted the LH and VP in separate experiments using a disconnection procedure since the LH-LHb and VP-LHb projections are both predominantly ipsilateral (Herkenham and Nauta, 1977). Here, ipsi- and contralateral lesions were made of the LHb and LH, or LHb and VP, so that in the ipsilaterally-lesioned rats the connection is still intact in one hemisphere, whereas in the contralaterally-lesioned rats the connection is lost in both hemispheres. This classic asymmetrical disconnection procedure has been used previously (Belin and Everitt, 2008; Peters et al., 2008; Bossert et al., 2012) and is based on the premise that learned behaviors can be maintained by an intact hemisphere (Gaffan et al., 1993; Setlow et al., 2002). I then investigated voluntary ethanol consumption, operant ethanol self-administration, and yohimbine-induced reinstatement of ethanol-seeking in these groups. My results suggest that the LH-LHb projection regulates voluntary ethanol consumption, whereas the VP-LHb is not necessary for mediating the tested ethanol-directed behaviors.

Materials and methods

Voluntary ethanol consumption was evaluated in SM-lesioned rats, LH-LHb-lesioned (ipsi- and contralateral) and VP-LHb-lesioned rats (ipsi- and

contralateral). Furthermore, I studied taste preference, operant ethanol self-administration, yohimbine-induced reinstatement in LH-LHb and VP-LHb groups. Finally, the effect of LH-LHb disconnection on ethanol-induced CTA was also investigated.

Subjects

Adult male Long-Evans rats were used as subjects across three separate experiments (400-500 g, Charles-River, Wilmington, MA). There were 22 rats (9 sham- and 13 SM-lesioned) for the SM experiment, 32 (16 ipsi- and 16 contralesioned) for the LH-LHb experiment, and 29 (14 ipsi- and 15 contra lesioned) for the VP-LHb experiment. Rats were single-housed in Plexiglas tub cages and maintained on a 12-hour (h) light/dark cycle. *Ad libitum* access to food and water was available throughout all experimental procedures. All procedures occurred during the light cycle (12:12 h), with lights on at 6 AM, unless otherwise stated. Experiments were approved by the University of Utah Animal Care and Use Committee and carried out in accordance with the *Guide for the Care and Use of Laboratory Animals (8th Edition).*

Drugs

Ethanol (Decon Labs, King of Prussia, PA) was prepared in filtered tap water (20% v/v) for the IEA experiment and in physiological saline for the CTA paradigm. Saccharin, quinine, and yohimbine (4 mg/ml) were prepared in distilled water (Sigma-Aldrich, St. Louis, MO). Ibotenic acid (10 µg/µl) was prepared in

physiological saline (Sigma-Aldrich, St. Louis, MO).

Surgical lesions

Electrolytic and excitotoxic lesions were conducted under isoflurane anesthesia (5% induction, 2% maintenance). Neo-Predef (a topical anesthetic), buprenorphine (0.06 mg/kg, IP), and penicillin (3 x 108 units/kg, IM) were also administered to provide analgesia and prevent infection, respectively. Chlorodiazepoxide (0.5 ml of 10 mg/ml) was injected IP after excitotoxic lesions to prevent seizures. Rats were placed in a flat-skull position in a stereotaxic apparatus, the skull exposed, and burr holes drilled above the target region.

For SM lesions, bilateral electrolytic lesions were produced by passing current (0.5 mA, 20 sec) through a stainless steel electrode (AM Systems, Sequim, WA) at the following coordinates: AP:-1.8mm, ML: ±0.9 mm, DV:-5.5 mm relative to bregma (Paxinos and Watson, 2007a). For sham lesions, electrodes were lowered 1 mm dorsal to the coordinates, but no current was passed. For LH-LHb and VP-LHb disconnection studies, unilateral lesions of each structure were made for all groups. For the ipsilateral groups, both the LHb and LH or VP were lesioned in the same hemisphere, allowing for functional communication of these structures in the alternate hemisphere. For the contralateral groups, the LHb and LH or VP were lesioned in opposite hemispheres, preventing functional communication of these structures. Excitotoxic lesions of the LH and VP were produced by infusing ibotenic acid (0.6 µl of 10 µg/µl) through a 31-gauge injector connected to polyethylene tubing

(PE50) connected to a 1-µl glass Hamilton syringe (Reno, NV) on a microinfusion pump (Harvard Apparatus 2000, Holliston, MA). For each infusion, a volume of 0.6 µl was injected over 4 min, and the injector was left in place for an additional 2 min to allow for diffusion of the drug. LH coordinates were: AP:-2.2 mm, ML: ±1.7 mm, DV: -9.1 mm. VP coordinates were: AP: 0.0 mm, ML: ±1.8 mm, DV: -8.4 mm, relative to bregma (Paxinos and Watson, 2007a). Electrolytic lesions of the LHb were produced by passing current (0.5 mA, 20 sec) through a stainless steel electrode (AM Systems, Sequim, WA) at the following coordinates: AP: -3.7 mm, ML: ±0.7 mm and DV: -5.6 mm (Paxinos and Watson, 2007a). The hemispheres lesioned were counterbalanced across rats.

Intermittent ethanol access (IEA) and taste preference

Voluntary ethanol consumption was monitored for 8 weeks using a two-bottle choice IEA paradigm for all 3 experiments. Briefly, one ethanol bottle and one water bottle were made available in home cages for three days each week, with one day off in between (as described in (Haack et al., 2014)). For the LH-LHb lesion experiment, 5 ipsi- and 4 contralaterally lesioned rats had to be eliminated due to misplacement of the LH or LHb lesion, resulting in a sample size of 11 ipsi- and 12 contralaterally lesioned rats for final analysis. For the VP-LHb lesion experiment, 4 ipsi- and 4 contralaterally lesioned rats were removed from analysis due to poor health or misplacement of the lesion, resulting in a final sample size of 10 ipsi- and 11 contralaterally lesioned rats. The same numbers of animals were used in subsequent experiments unless otherwise mentioned.

Total ethanol and water consumed, as well as ethanol preference over water [ethanol intake/total fluid intake (water +ethanol intake)] was calculated for each ethanol drinking session, for a total of 24 drinking sessions. Water intake, ethanol intake and ethanol preference were averaged for each week. Food was available *ad libitum* and food intake was monitored weekly for all rats. Rats were weighed weekly.

Following IEA, taste preference and taste aversion were also assessed using two-bottle choice paradigms wherein intake of water was compared to that of saccharin and quinine solutions in LH-LHb and VP-LHb groups. Quinine intake was measured first (6 consecutive daily sessions), followed by saccharin intake (a further 6 sessions), as previously described (Haack et al., 2014).

Operant responding for ethanol

Operant responding for ethanol was investigated in LH-LHb and VP-LHb rats following the IEA procedure. Training occurred in eight standard Med Associates chambers (St. Albans, VT), as previously described (Haack et al., 2014). Briefly, responding on the active lever resulted in retraction of the lever, extinguishing of the cue light, activation of the syringe pump, and delivery of ethanol in the receptacle. Initially, only an active lever was present during training and every lever response was reinforced with 0.1 ml of 20% ethanol (i.e., FR-1 schedule). After an initial overnight session, rats were trained daily for 60 min each session until they responded at stable levels (less than 20% variability across two sessions). Then, the reinforcement schedule was set at FR-3 (i.e.,

every third lever press was reinforced). After stable responding on this paradigm was attained, the inactive lever was introduced, on which pressing was recorded, but not reinforced. The number of active lever presses across the last three sessions was averaged for each rat.

Extinction and reinstatement of ethanol-seeking

Next, extinction of operant responding for ethanol was examined. Extinction sessions were identical to the training sessions except that the syringe containing the ethanol was removed from the syringe pump. Thus, responding on the active lever resulted in retraction of the lever, extinguishing of the cue light, and activation of the syringe pump, but no ethanol delivery. Extinction sessions were conducted daily. Once responding was reduced to a total of 15 or fewer active lever presses for three consecutive sessions, the rats were tested for yohimbine-induced reinstatement.

Yohimbine (2 mg/kg) or vehicle solution was administered (IP) 30 min prior to testing responding in extinction. The dose and timing of yohimbine was based on our previous study (Haack et al., 2014), except that the session length during reinstatement was increased to 90 minutes to ensure reliable and robust yohimbine-induced reinstatement in control rats (Gill et al., 2013). To minimize variability in response rates, each animal was injected twice with yohimbine and twice with water, in counter-balanced order across rats. Each test session was separated by an extinction session to ensure reinstated responding was reduced back to extinction levels. Responses were then averaged for the two tests for

each rat. For VP-LHb, one ipsi- and one contralesioned rat did not extinguish and hence could not be tested for yohimbine-induced reinstatement, resulting in a final sample size of 9 ipsi- and 10 contralaterally lesioned rats.

Ethanol-induced conditioned taste aversion (CTA)

After reinstatement testing, LH-LHb-lesioned rats were subjected to a CTA paradigm based on a previously published protocol (Rinker et al., 2011). In brief, rats were initially water deprived for 24 hours, and then received 20 minute access to tap water in their home cage for three sessions. Rats were then given access to saccharin (0.125% in tap water) for 20 minutes in their home cages, and consumption of that saccharin was measured. Rats were then divided into four groups, matched for saccharin consumption: ipsi-vehicle (n=5), ipsi-ethanol (n=6), contra-vehicle (n=7) and contra-ethanol (n=5). Rats were then injected with 1.5 g/kg body weight of 20% v/v ethanol (IP) or an equivalent volume of vehicle (saline). On the following two days, rats received 20-minute access to tap water in their home cages, which was not paired with an injection. This three-day cycle (saccharin paired with injection followed by two days of water not paired with injection) was repeated three times (i.e., three conditioning trials). Then, CTA was extinguished by exposing the rats to the same cycle, with the only exception being that saccharin access was no longer paired with an injection. This three-day cycle continued until saccharin consumption returned to the pre-CTA level. One contralaterally lesioned rat had to be eliminated from analysis due to profound neophobia to the saccharin solution, resulting in a final sample

size of 11 ipsi- and 11 contralaterally lesioned rats.

Verification of lesions

Lesions sites were verified after testing. Rats were deeply anesthetized with sodium pentobarbital (140 mg/kg) and transcardially perfused with saline, followed by 4 % paraformaldehyde. The brains were removed, cryoprotected, and then cut on a freezing microtome (45-µm thickness). For electrolytic lesions of SM, sections were mounted onto glass slides, dried, and stained with cresyl violet before being dehydrated and cover slipped. For lesions of LHb and LH or VP, brain sections were immuno-stained for neuronal nuclei (NeuN) based on a previously published protocol (Furlong and Carrive, 2007). Lesions were verified using a light microscope, and plotted on templates modified from a rat brain atlas (Paxinos and Watson, 2007a).

Retrograde and c-Fos study

In order to confirm the disconnection of LHb and its afferents (VP and LH), a separate group of 6 animals was used to localize LH and VP projections using the retrograde tracer, cholera-toxin b (CTb). CTb was targeted unilaterally at LHb (40 nl; -3.6 AP, -5.1 DV, ±0.7 ML mm from bregma) during stereotaxic surgery, and the animals allowed one week to recover. To further inform the yohimbine reinstatement studies, I examined the recruitment of LH and VP and quantified CTb, c-Fos and double-labelled c-Fos/CTb in these structures after yohimbine exposure. Animals were then injected with either yohimbine (2.5 mg/kg, IP) or

vehicle 2 hrs prior to sacrifice, and the brains immunoprocessed for c-Fos and CTb. Total counts were averaged across three sections for each animal, and then averaged for each group. CTb labelling was also depicted on brain atlas templates for all 6 animals, where each CTb-labelled neuron was represented as a brown dot.

Statistical analyses

Voluntary ethanol consumption, ethanol preference, water intake, and total fluid intake during IEA were analyzed using 2-way repeated-measures (RM) analysis of variance (ANOVA) with lesion condition (sham or lesioned for SM experiments, and ipsi or contra in LH-LHb and VP-LHb experiments) and week as the two factors. Taste preference, extinction of ethanol-seeking, and yohimbine-induced reinstatement were also analyzed using 2-way RM ANOVA with lesion condition as one factor and tastant concentration, extinction session, or drug (vehicle or yohimbine) as the second factor. CTA results were analyzed using 3-way RM ANOVA with lesion condition and drug as between factors, and trial as within factor. Differences between groups during operant ethanol self-administration were analyzed using Student's t-test. Average counts of CTb, c-Fos and c-Fos/CTb were analyzed using a two factor ANOVA where treatment (yohimbine and vehicle) and region (LH and VP) served as the two factors. JMP Pro 11 (SAS Institute Inc., Cary, NC) was the statistical software used to carry out analyses. Analyses were considered significant when $p<0.05$. Data is shown as mean ± S.E.M.

Results

Effects of SM lesion on intermittent ethanol access

Average intake of ethanol each week was calculated by averaging the ethanol intake for the three days of the week on which ethanol and water were available for each group. As can be seen in Figure 5.1a, ethanol intake progressively increased across eight weeks of IEA in both sham- and SM-lesioned rats (Figure 5.1a; significant main effect of week, $F(2.6,51.3)=27.9$, $p<0.0001$). However, the SM-lesioned rats escalated their ethanol intake faster than sham-lesioned rats, and reached higher levels of ethanol intake at the end of 8 weeks (significant interaction of lesion condition and week, $F(2.6,51.3)=3.3$, $p<0.05$). Posthoc tests showed that SM-lesioned rats drank more ethanol starting from week 5 and through week 8. There was also a significant ethanol preference over water for SM-versus sham-lesioned group (Figure 5.1b; significant main effect of week, $F(3.2,65)=33.8$, $p<0.0001$, significant interaction of lesion condition and week, $F(3.2,65)=3.4$, $p<0.05$). Posthoc tests showed that SM-lesioned rats had a higher preference for ethanol at week 7.

Water intake declined in both sham-and SM-lesioned rats as the IEA progressed (Figure 5.1c; significant main effect of week, $F(6.3,125.3)=27.9$, $p<0.0001$), however, SM-lesioned rats reduced water intake to a greater degree than the sham-lesioned rats during the IEA (Fig 5.1c; significant interaction of lesion condition and week, $F(6.3,125.3)=3.2$, $p<0.01$, posthoc significance at week 7). However, total fluid intake (ethanol plus water) was not statistically different between sham- and SM-lesioned groups, suggesting that SM-lesioned

group reduced water intake to compensate for the increase in ethanol intake (Figure 5.1d; no significant main effect of lesion condition, $F(1,20)=0.4$, $p=0.54$, no significant interaction of lesion condition and week, $F(4.6,93.1)=1.0$, $p=0.4$). Finally, there was no difference between groups for body weight across the duration of IEA, indicating that the SM lesions did not alter body weight (data not shown, no significant main effect of lesion condition, $F(1,20)=0.3$, $p=0.56$, significant main effect of week, $F(1.5,30.2)=401.8$, $p<0.0001$, significant interaction of lesion condition and week, $F(1.5,30.2)=3.6$, $p=0.05$ but no posthoc significance).

Effects of LH-LHb lesion on intermittent ethanol access

Ethanol intake increased progressively for both rats with ipsilateral LH-LHb lesions and rats with contralateral LH-LHb lesions (Figure 5.2a; significant main effect of week, $F(1.8,37.9)=11.7$, $p<0.0005$). However, contra- rats consumed significantly more ethanol than ipsilaterally lesioned rats overall (significant interaction of lesion condition and week, $F(1.8,37.9)=3.8$, $p<0.05$, posthoc significance from week 6 through week 8). Contralaterally lesioned rats also had a higher ethanol preference than ipsilaterally lesioned (Figure 5.2b; significant interaction of lesion condition and week, $F(2.3,48.7)=3.9$, $p<0.05$, significant main effect of week, $F(2.3,48.7)=7.4$, $p<0.001$, posthoc significance from week six through week 8).

Water intake of the contra- rats was lower than that of ipsilaterally lesioned rats (Figure 5.2c; significant interaction of lesion condition and week,

$F(2.8,58.1)=3.1$, $p<0.05$, posthoc significance at weeks 6, 7 and 8). However, total fluid intake (ethanol plus water) did not differ between the two groups (Figure 5.2d; no significant interaction of lesion condition and week, $F(2.4,50.8)=0.7$, $p=0.54$ and no significant main effect of lesion, $F(1,21)=0.5$, $p=0.5$), suggesting that the contralaterally lesioned rats reduced water intake to compensate for the increase in ethanol intake. Finally, there were no statistically significant differences in body weight or food intake across the duration of IEA between ipsi- and contralaterally lesioned rats (body weight: no significant interaction of lesion condition and week, $F(1.5,32.1)=0.3$, $p=0.67$, no significant main effect of lesion condition, $F(1,21)=0.08$, $p=0.77$ but main effect of week, $F(1.5,32.1)=169.9$, $p<0.0001$; food intake: no significant interaction of lesion condition and week, $F(2.8,59.9)=1.8$, $p=0.16$ and no significant main effect of lesion condition, $F(1,21)=0.01$, $p=0.9$ but main effect of week, $F(2.8,59.9)=3$, $p<0.05$).

Effects of LH-LHb lesion on taste preference

I evaluated taste preference in LH-LHb-lesioned rats to investigate whether taste preference influenced ethanol intake in the IEA paradigm. Quinine preference decreased for both groups with increasing quinine concentration (Figure 5.3a; significant main effect of quinine concentration, $F(3.1,65.2)=82.2$, $p<0.0001$). However, lesion condition did not have an effect on quinine preference (no significant interaction of lesion condition and quinine concentration, $F(3.1,65.2)=1.3$, $p=0.26$, no significant main effect of lesion

condition, F(1,21)=1.5, p=0.23,). Saccharin preference increased with increasing saccharin concentration for both groups, but again lesion condition had no effect on saccharin preference (Figure 5.3b; significant main effect of saccharin concentration, F(3.6,75.9)=126.2, p<0.0001, no significant interaction of lesion condition and saccharin concentration, F(3.6,75.9)=0.68, p=0.59 and no significant main effect of lesion condition, F(1,21)=0.1, p=0.75).

Effects of LH-LHb lesion on operant ethanol self-administration, extinction and, yohimbine-induced reinstatement

Figure 5.4a shows the average lever presses made for each group during the last three ethanol self-administration sessions, and Figure 5.4b shows lever presses for each session during extinction when ethanol was withheld. There were no differences between rats with ipsilateral and contralateral LH-LHb lesions with respect to the number of lever presses during self-administration (Figure 5.4a; t=-0.83, p=0.21), or in the rate of extinction of this response (Figure 5.4b; main effect of extinction session, F(2,42.7)=21.7, p<0.0001; no significant effect of lesion condition, F(1,21)=0.2, p=0.67, no significant interaction of lesion condition and extinction session, F(2,42.7)=0.2, p=0.81). Figure 5.4c shows the yohimbine-induced reinstatement test, where it can be seen that both the ipsi- and contralaterally lesioned groups showed yohimbine-induced reinstatement of lever pressing (significant main effect of treatment, F(1,21)=15.1, p<0.001, no significant main effect of lesion condition, F(1,21)=0.07, p=0.79, no significant interaction of lesion condition and treatment, F(1,21)=0.2; p=0.62).

Effects of LH-LHb lesion on ethanol-induced CTA

To investigate whether the increased ethanol consumption seen in contra-lesioned rats was due to attenuation of ethanol-induced aversive learning, the rats were subjected to a CTA paradigm. As can be seen in Figure 5.5, ethanol injection conditioned a robust aversion to saccharin in both ipsi- and contralaterally-lesioned rats (significant main effect of ethanol treatment, $F(1,18)=46.6$, $p<0.0001$; significant main effect of trial, $F(9.3,167.5)=17.8$, $p<0.0001$;significant interaction of ethanol treatment and trial, $F(9.3,167.5)=23.3$, $p<0.0001$). There was no difference between lesion groups in conditioning or extinction of CTA, as both groups showed a similar decrease in saccharin intake when it was paired with alcohol injection and a similar recovery of saccharin consumption when it was no longer paired with ethanol injection (Figure 5.5; no significant main effect of lesion condition, $F(1,18)=0.6$, $p=0.46$; no significant interaction of ethanol treatment and lesion condition, $F(1,18)=0.4$, $p=0.55$;, no significant interaction of trial and lesion condition, $F(9.3,167.5)=0.5$, $p=0.85$; no significant 3-way interaction, $F(9.3,167.5)=0.9$, $p=0.54$).

Effects of VP-LHb lesion on intermittent ethanol access,
taste preference, operant ethanol self-administration,
extinction, and yohimbine-induced reinstatement

I similarly examined the effects of ipsilateral and contralateral lesions of the VP-LHb projection. In these studies, both rats with ipsilateral VP-LHb lesions and contralateral VP-LHb lesions increased their ethanol intake as the IEA

progressed (Figure 5.6a; significant effect of week, $F(2.9,55)=17.7$, $p<0.0001$). However, there was no difference between the lesion groups, indicating that ablating the VP-LHb connection did not alter ethanol intake (no significant main effect of lesion condition, $F(1,19)=0.3$, $p=0.57$; no significant interaction of lesion condition and week $F(2.9,55)=0.4$, $p=0.74$).

Water intake was not different between groups, with both groups drinking about 24 ml in week 1 (start) and about 14 ml in week 8 (end; data not shown; significant main effect of week, $F(3.6,68.9)=19.6$, $p<0.0001$; no significant main effect of lesion condition, $F(1,19)=0.5$, $p=0.49$; no significant interaction of lesion condition and week, $F(3.6,68.9)=0.4$, $p=0.76$). VP-LHb disconnection also had no significant effect on body weight (data not shown, significant main effect of week, $F(2.3,45.5)=258.1$, $p<0.0001$;no significant main effect of lesion condition, $F(1,19)=0.4$, $p=0.43$; significant interaction of lesion condition and week, $F(2.3,45.5)=4.7$, $p<0.01$, but no posthoc differences). Finally, the groups did not differ in food intake (significant main effect of week, $F(3.3,62.1)=5.8$, $p<0.005$; no significant main effect of lesion condition, $F(1,19)=0.6$, $p=0.44$; no significant interaction of lesion condition and week, $F(3.3,62.1)=1$, $p=0.38$).

There also were no statistically significant differences between the ipsi- and contralaterally lesioned groups in the number of lever presses made during operant ethanol self-administration (Figure 5.6b; $t=-0.2$, $p=0.85$), the rate of extinction of ethanol-seeking (Figure 5.6c; significant main effect of extinction session, $F(2.5,48.3)=10.8$, $p<0.0001$; no significant main effect of lesion condition, $F(1,19)=0.03$, $p=0.86$; no significant interaction of lesion condition and

extinction session, $F(2.5,48.3=0.7$, $p=0.5)$ or in yohimbine-induced reinstatement of ethanol-seeking (Figure 5.6d; significant main effect of treatment, $F(1,17)=10.6$, $p<0.005$; no significant main effect of lesion condition, $F(1,17)=1.4$,$p=0.25$; no significant interaction of lesion condition and treatment, $F(1,17)=0.6$, $p=0.45$).

Both rats with ipsilateral and contralateral VP-LHb lesions showed increasing aversion to increasing concentrations of quinine (Figure 5.7a; significant main effect of concentration, $F(3.9,73.8)=64.6$, $p<0.0001$). Overall, the contralaterally-lesioned rats had higher preference for quinine (significant main effect of lesion condition, $F(1,19)=6.1$, $p<0.05$; nonsignificant trend for an interaction of lesion condition and concentration, $F(3.9,73.8)=2.4$, $p=0.06$). Saccharin preference increased as a function of concentration in ipsi- and contralaterally lesioned rats (Figure 5.7b; significant main effect of concentration, $F(3.4,64.1)=89.4$, $p<0.0001$). However, the increase in saccharin preference was not dependent on lesion condition (significant interaction of lesion condition and lesion condition, $F(3.4,64.1)=2.7$, $p<0.05$, but no posthoc significance).

Retrograde and c-Fos study

Figure 5.8 shows the CTb injection sites of the 6 animals, and back-labelling of CTb in the LH and VP. The location of the CTb coincides well with the lesion placements of the previous experiments suggesting that the disconnections were appropriate. The average numbers of CTb back-labeling per section is shown in Table 5.1. Analysis revealed that the number of CTb was

greater for the LH than the VP ($F(1,4)=7.47$, $p=0.05$), but was not different between treatment groups ($F(1,4)=0.29$, $p=0.62$).

Very few CTb projection neurons were recruited by yohimbine, despite the presence of c-Fos in both LH and VP (Figure 5.8e and 5.8f, Table 5.1). Specifically, c-Fos was greater following yohimbine versus control treatment ($F(1.4)=11.90$, $p=0.03$) and for the LH versus VP ($F(1,4)=102.01$, $p<0.01$). Importantly, there was also a treatment by region interaction ($F(1,4)=27.04$, $p=0.01$). Posthoc analysis revealed that there was greater c-Fos following yohimbine treatment compared to control treatment in LH ($F(1,4)=15.75$, $p=0.03$) but not in VP ($F(1,4)=11.90$, $p=0.09$). Analysis of double-labeled neurons confirmed that there were no differences between treatment groups, regions and no interaction ($F(1,4)<0.50$, $p>0.52$ for all contrasts).

Discussion

The LHb plays a critical role in voluntary ethanol consumption, operant ethanol self-administration and yohimbine-induced reinstatement of ethanol-seeking (Haack et al., 2014). However, whether afferent input to the LHb is necessary for its effects on ethanol-directed behaviors is unknown. Therefore, I first determined if afferent inputs are at all necessary for effects of LHb on ethanol consumption by lesioning the SM. I found increased voluntary ethanol consumption in SM-lesioned rats (Figures 5.1a and 5.1b), suggesting that one or more inputs to LHb are important for voluntary consumption. I then further explored the LH and VP inputs to LHb using a disconnection procedure. I found

that disconnection of the LH-LHb pathway was associated with escalated ethanol consumption, whereas disconnection of the VP-LHb pathway was not. These results suggest that the LH-LHb projection regulates voluntary ethanol consumption, but the VP-LHb projection does not. Finally, while my previous study found that LHb also regulated operant ethanol self-administration, yohimbine-induced reinstatement of ethanol-seeking and ethanol-induced CTA (Chapter 2), the present results failed to support a role of either the LH or the VP afferents to LHb in those behaviors, suggesting that other inputs to LHb are likely to be involved in mediating the role of the LHb in these behaviors.

Effects of LH-LHb and VP-LHb disconnection on voluntary ethanol consumption

The results show that contralaterally lesioned rats drink more ethanol and have a higher ethanol preference in the IEA paradigm than the ipsilaterally lesioned rats in the LH-LHb group (Figures 5.2a and 5.2b). Given that I used the classical disconnection strategy, these results suggest that the LH projection to the LHb is necessary for regulating voluntary ethanol consumption. This is the first study providing evidence for a functional role for the LH-LHb projection in controlling ethanol intake. The LH is known for its role in consummatory behaviors and has previously been shown to be involved in regulating ethanol consumption (Chen et al., 2013; Chen et al., 2014; Marchant et al., 2014; Navarro et al., 2015). For example, a subpopulation of LH neurons bi-directionally regulates ethanol consumption in mice (Navarro et al., 2015).

Further, DA and opioid transmission in the LH also regulate ethanol consumption (Chen et al., 2013; Chen et al., 2014). Our results suggest that LH may exert these effects on ethanol consumption via its projection to the LHb.

The actions of ethanol on LHb neurons may be mediated by the LH-LHb projection. Acute ethanol increases LHb neuronal firing by enhancing presynaptic glutamate release onto LHb neurons; thus, the effects of ethanol on LHb neurons require intact excitatory input to the LHb (Zuo et al., 2015). The LH provides a major glutamatergic projection to the LHb neurons (Poller et al., 2013). Therefore, it is reasonable to speculate that ethanol increases the excitatory drive of LH afferents onto LHb neurons, which then increases inhibition on downstream DA and 5-HT neurons (Poller et al., 2013), limiting ethanol intake. When the LH-LHb projection is lesioned, ethanol can no longer enhance LHb neuronal firing, which leads to disinhibition of ethanol intake.

I previously showed that escalation of ethanol consumption following LHb lesion was likely to be due to reduced aversion to ethanol. Specifically, LHb lesions attenuated ethanol-induced aversive conditioning in a CTA paradigm (Haack et al., 2014). However, in the current study, the disconnection of the LH-LHb pathway did not affect CTA (Figure 5.5). This finding suggests that a different mechanism is responsible for the escalated consumption observed in the absence of LH input to the LHb. The nature of this mechanism, however, is presently unclear. The LH does play a role in consumption and maintenance of body weight, and recent evidence suggests that the LH is involved in consumption behavior directed at any stimulus (Navarro et al., 2015). Thus, in

the present study, ethanol might have been consumed for its caloric value. However, given that contralaterally lesioned rats were of similar weight and did not consume more food or fluid than ipsilaterally lesioned rats during the IEA procedure, it seems unlikely that the greater ethanol intake was driven by caloric value. A second possibility is that a difference in taste might be the basis for the greater ethanol consumption. However, this also seems unlikely as a potential mechanism, as the taste preference for quinine and saccharin was not different between LH-LHb ipsi- and contralesioned rats. Finally, it is possible that altered anxiety levels might contribute to the greater ethanol intake in the rats in whom the LH-LHb connectivity was lost. The LH and LHb both play important roles in regulating anxiety, although a role for the LH-LHb projection in anxiety-related behaviors has not been established (Hakvoort Schwerdtfeger and Menard, 2008; Gill et al., 2013; Kim et al., 2013). Given that anxiety is an important factor in controlling ethanol intake (Spanagel et al., 1995; Henniger et al., 2002), it is possible that disconnection of the LH-LHb projection alters anxiety levels, which then increases ethanol intake. Additional studies investigating the causal role of the LH-LHb projection in anxiety-related behaviors and whether such behaviors impact ethanol consumption are therefore required.

In addition to the LH-LHb projection, we investigated the role of the VP-LHb projection in ethanol-directed behaviors, because of evidence that reward-related signals can be relayed to the LHb via inhibitory inputs from the VP (Hong and Hikosaka, 2013). Specifically, LHb neurons are inhibited by stimulation in the VP (Hong et al., 2011). Given that VP neurons show positive value coding

(Tachibana and Hikosaka, 2012), this signal could be converted to negative value coding in the LHb via the inhibitory VP-LHb connection, making the VP an important source of reward value signal to the LHb. This finding, coupled with results supporting the role of opioid transmission in the VP in mediating ethanol reward (Kemppainen et al., 2012), made the VP a good candidate for mediating the effects of the LHb on voluntary ethanol consumption. However, in contrast to the LH-LHb disconnection, the VP-LHb disconnection did not alter ethanol consumption.

Although the VP-LHb pathway did not appear to regulate ethanol consumption, this pathway may play a role in consumption of aversive tastes. The VP is known for its role in mediating taste-reactivity and palatability-related intake (Smith et al., 2009). Specifically, GABA-A agonists in the VP reduce quinine intake (Shimura et al., 2006). In addition, the habenula has been implicated in mediating quinine aversion (Donovick et al., 1969; Donovick et al., 1970). In the present work, I found that disconnection of the VP-LHb pathway in rats is associated with those rats having a greater preference for quinine, suggesting that VP may mediate consumption of bitter tastants via the LHb.

Effects of LH-LHb and VP-LHb disconnection on operant ethanol self-administration and, yohimbine-induced reinstatement

In addition to the effect of LHb lesions on ethanol consumption, it was also previously found that LHb lesions increase operant ethanol self-administration

and block yohimbine-induced reinstatement of ethanol-seeking (Haack et al., 2014). In the present work, however, neither LH-LHb nor VP-LHb disconnection had any effect on these behaviors. These results suggest that LH and VP afferents to the LHb are not necessary for mediating effects of the LHb on operant ethanol self-administration and yohimbine-induced reinstatement of ethanol-seeking, and that alternate inputs to the LHb may be important. The analysis of c-Fos and CTb suggests that, despite the recruitment of the LH during yohimbine exposure, LH neurons projecting to the LHb are not recruited. VP neurons projecting to the LHb were also not recruited. This study provides further support for our findings that LH-LHb and VP-LHb are not important for yohimbine-induced reinstatement. A potential afferent candidate that could be critical for the effects of the LHb on yohimbine-induced reinstatement of ethanol-seeking is the BNST, given its role in yohimbine-induced reinstatement of cocaine-seeking and stress-induced behaviors (Dong and Swanson, 2006; Buffalari and See, 2011).

My findings also suggest that operant ethanol self-administration vs. free home cage access involve different neural circuits, given that LH-LHb disconnection increased ethanol consumption in the home cage, but did not alter operant ethanol self-administration behavior. Operant self-administration examines appetitive or “seeking” behavior, whereas free access to the reinforcer in the home cage examines consummatory or “taking” behavior. The neural correlates of these two behaviors are known to be different. For example, a DA D2 receptor antagonist reduces ethanol-seeking, but not ethanol-taking. Further,

FDA-approved drugs for ethanol dependence, such as acamprostate and naltrexone, reduce ethanol-taking without affecting ethanol-seeking (Brown et al., 1982; Czachowski et al., 2001; Czachowski et al., 2002). Therefore, the LHb may be a point of convergence for both ethanol-taking and ethanol-seeking behaviors, since LHb lesions increase both voluntary ethanol consumption (taking) and operant ethanol self-administration (seeking) (Haack et al., 2014), with different afferents regulating the different aspects of LHb control over ethanol-related behaviors.

Conclusions

In summary, this study suggests that afferent input to the LHb is critical in mediating effects of LHb on voluntary ethanol consumption. Specifically, the excitatory LH-LHb input seems to be critical in this regard. The results further suggest that the LH-LHb input is not necessary for driving the role of the LHb in other ethanol-directed behaviors. Furthermore, these results suggest that the VP-LHb input is not necessary for mediating LHb effects on any ethanol-directed behaviors. This study is thus the first to differentiate specific functional roles of key afferents to the LHb with respect to ethanol-associated behaviors.

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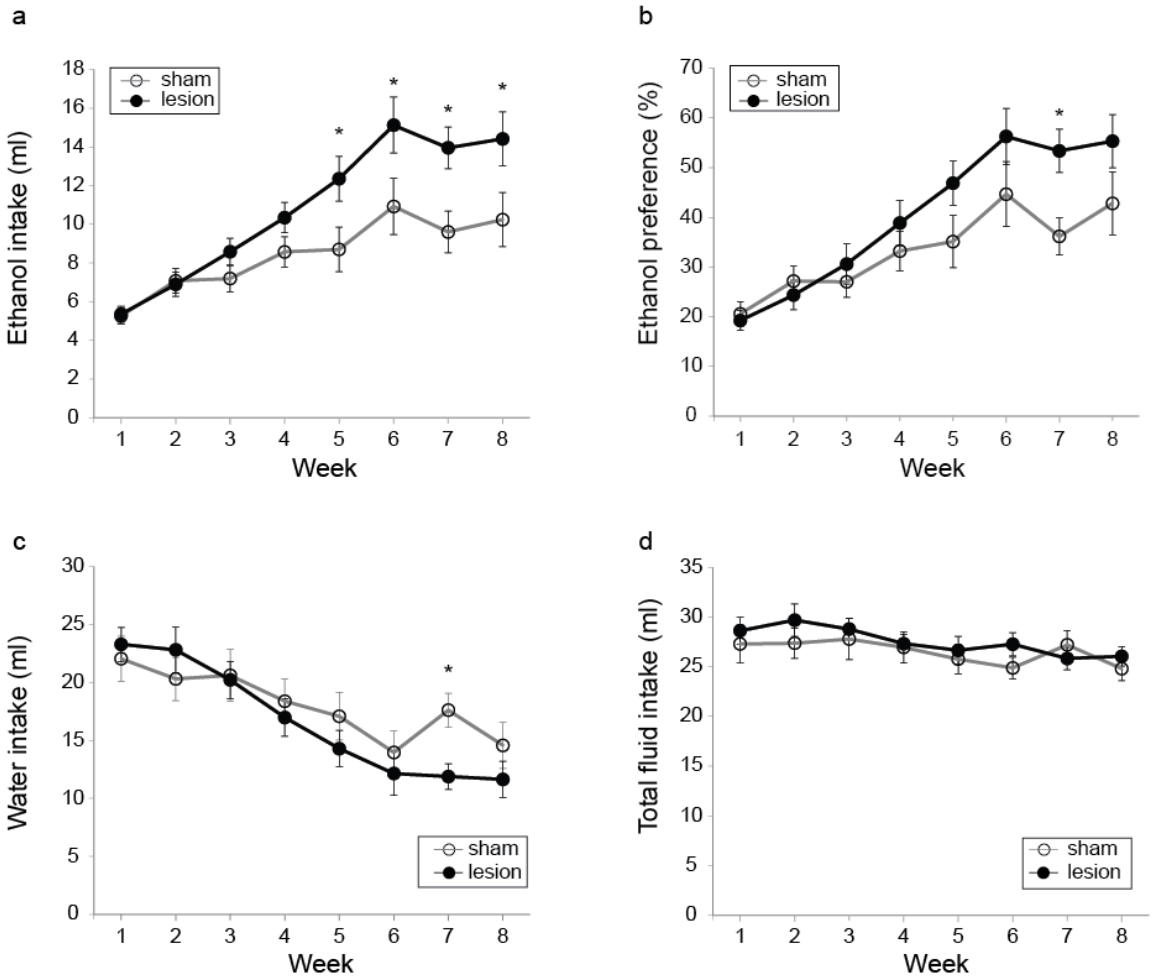

Figure 5.1 Effect of SM lesion on voluntary ethanol consumption (a) SM lesions increase voluntary ethanol consumption in the IEA paradigm. Weekly intake was calculated by averaging the ethanol intake on 3 days of the week where ethanol and water were available. Sham- and SM-lesioned rats are indicated by open and closed circles, respectively, unless otherwise indicated (b) SM lesions increase preference for ethanol in the IEA paradigm (c) SM lesions decrease water intake during IEA (d) Total fluid intake does not differ between the two groups. Data are represented as mean ± S.E.M. Asterisk indicates posthoc significance ($p<0.05$).

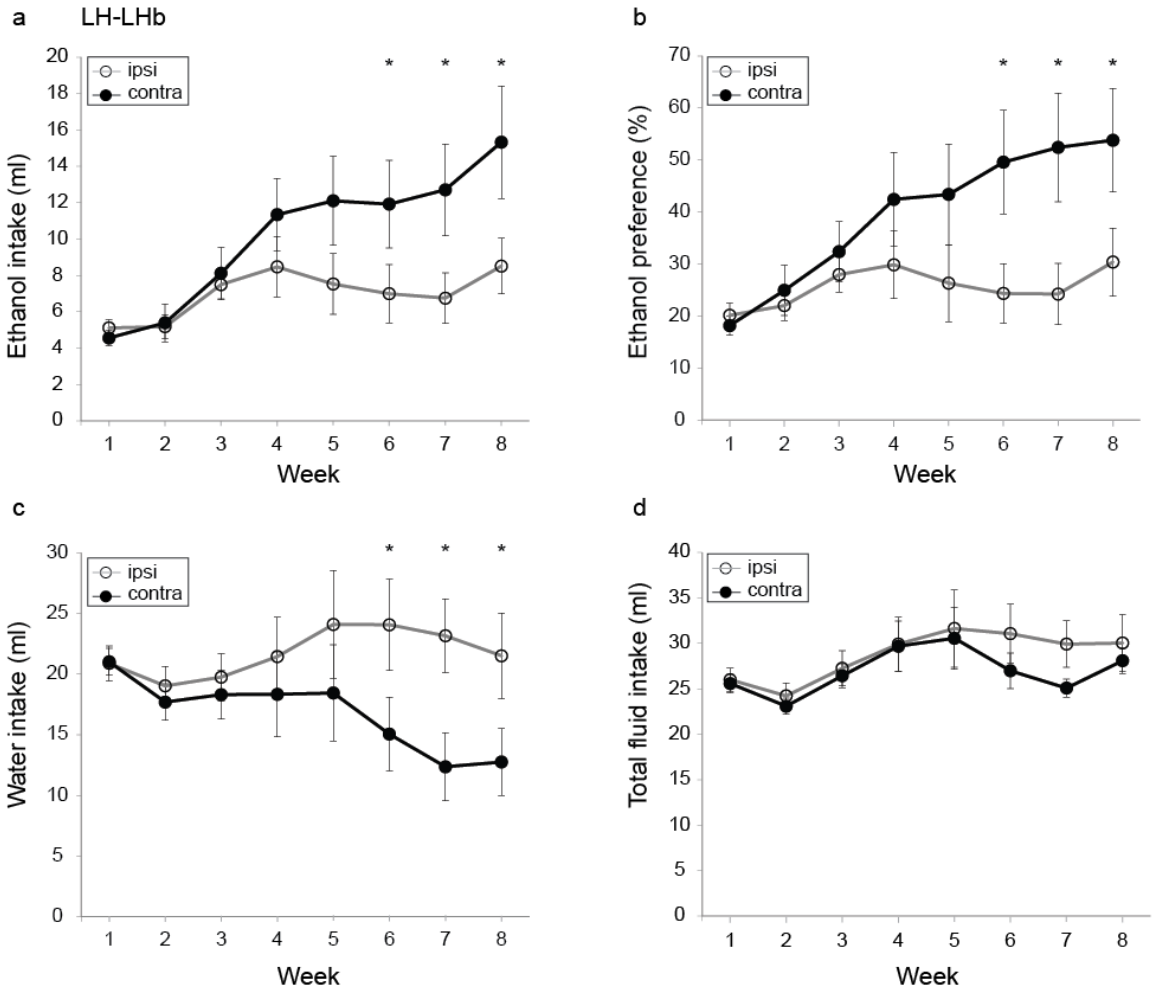

Figure 5.2 Effect of LH-LHb disconnection on voluntary ethanol consumption. Lesioning LH-LHb input increases (a) voluntary ethanol consumption and (b) preference in IEA paradigm (c) decreases water consumption (d) does not alter total fluid intake in the IEA paradigm. Open and closed circles represent ipsi- and contralesioned rats, respectively. Data are represented as mean ± S.E.M. Asterisk indicates posthoc significance ($p<0.05$).

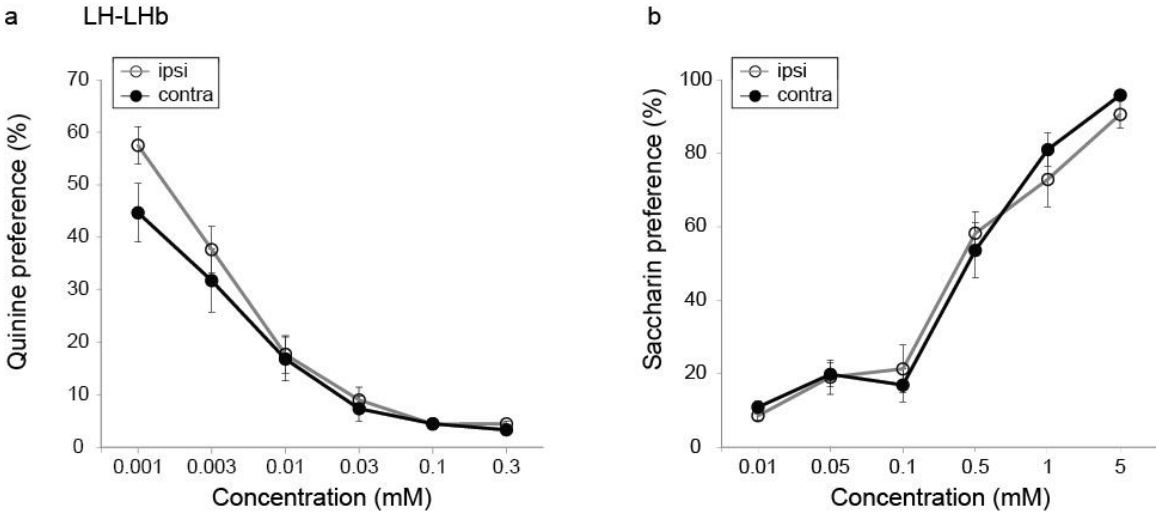

Figure 5.3 Effect of LH-LHb disconnection on taste preference. Lesioning LH-LHb input does not alter (a) quinine aversion or (b) saccharin preference.

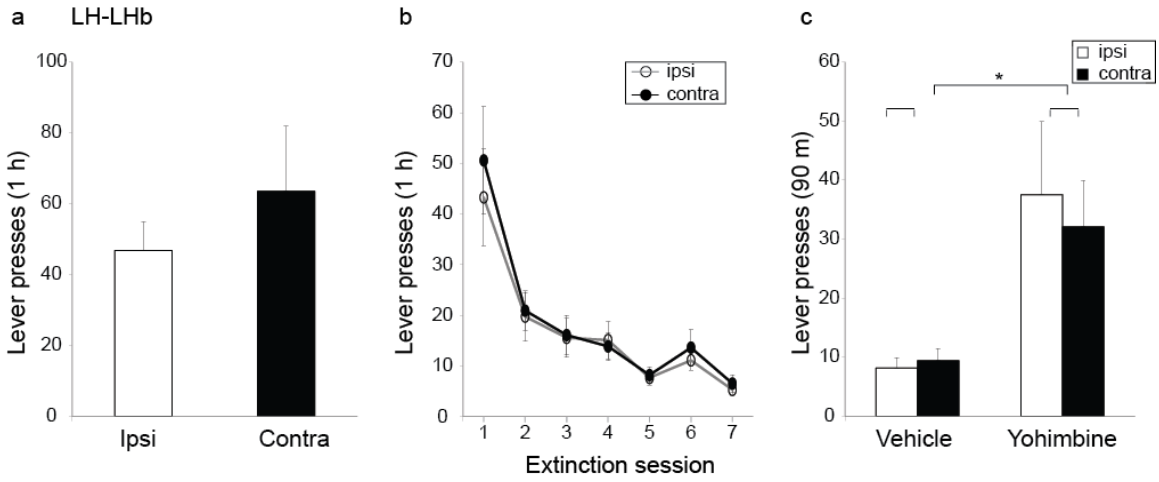

Figure 5.4 Effect of LH-LHb disconnection on operant ethanol behaviors. Lesioning LH-LHb input does not alter (a) operant ethanol self-administration, (b) rate of extinction of ethanol-seeking or (c) yohimbine-induced reinstatement of ethanol-seeking. Ipsi- and contralesioned rats are shown as open and closed bars respectively in (a) and (c), and as open and closed circles in (b). Data is represented as mean ± S.E.M. Asterisk indicates main effect of drug (yohimbine) in (c).

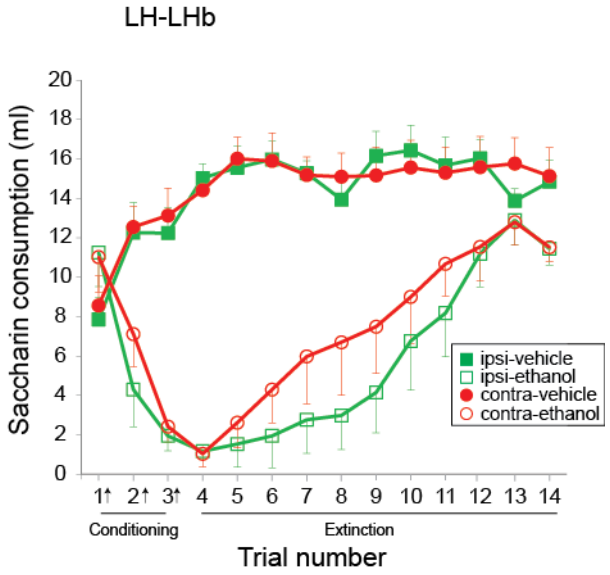

Figure 5.5 Effect of LH-LHb disconnection on ethanol-induced CTA. Lesioning LH-LHb input does not alter ethanol-induced CTA. Ipsi- and contralesioned rats show robust conditioned aversion to ethanol and similar rates of extinction of CTA. Arrows (x-axis) indicate trials in which saccharin consumption was paired with ethanol injection. Closed and open symbols indicate treatment with vehicle and ethanol, respectively. Squares represent ipsi- and circles represent contralesioned rats. Data are represented as mean ± S.E.M.

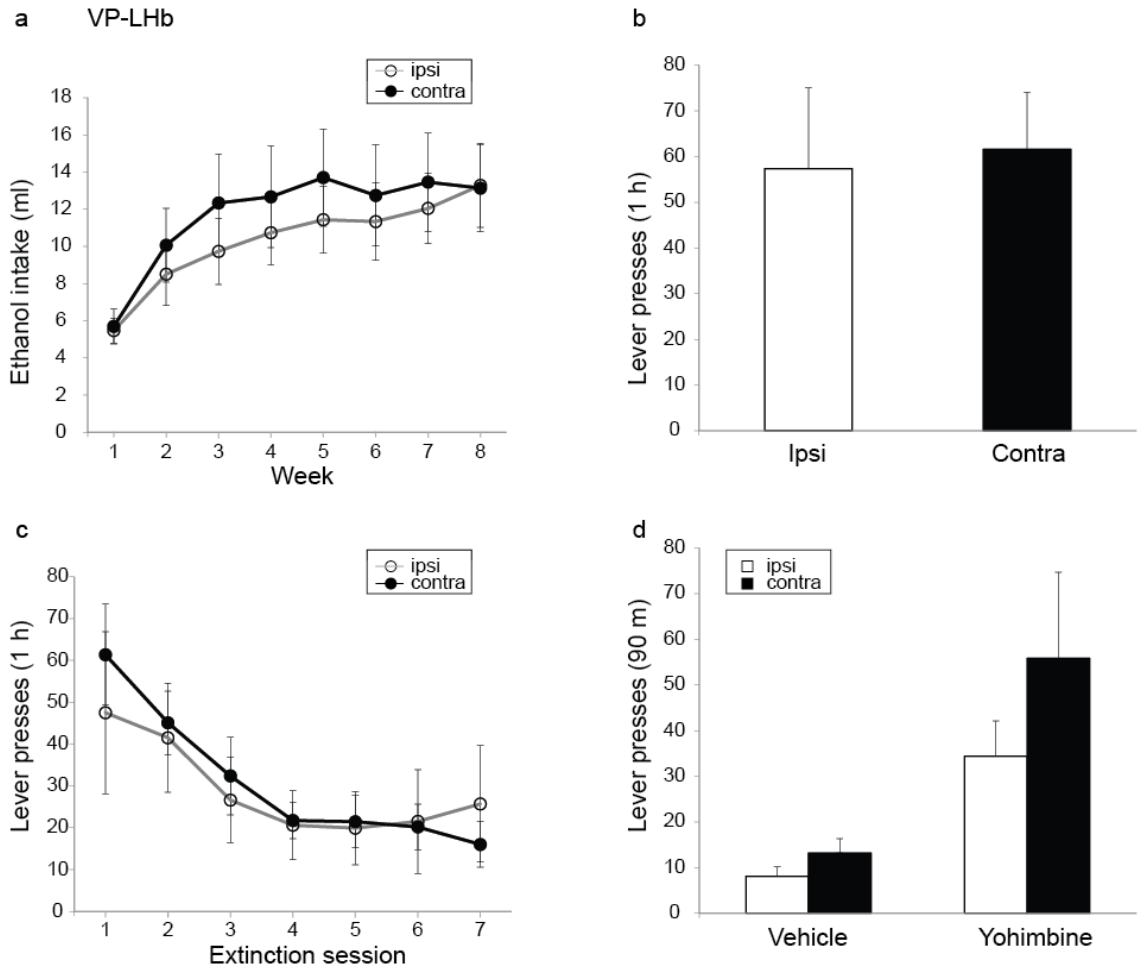

Figure 5.6 Effect of VP-LHb disconnection on ethanol-directed behaviors. Lesioning VP-LHb input does not alter (a) ethanol consumption in IEA paradigm or (b) operant self-administration of ethanol (c) rate of extinction of ethanol-seeking or (d) yohimbine-induced reinstatement of ethanol-seeking. Ipsi- and contralesioned rats are represented as open and closed circles, respectively, in (a) and (c), and as open and closed-bars, respectively, in (b) and (d). Data are represented as mean ± S.E.M.

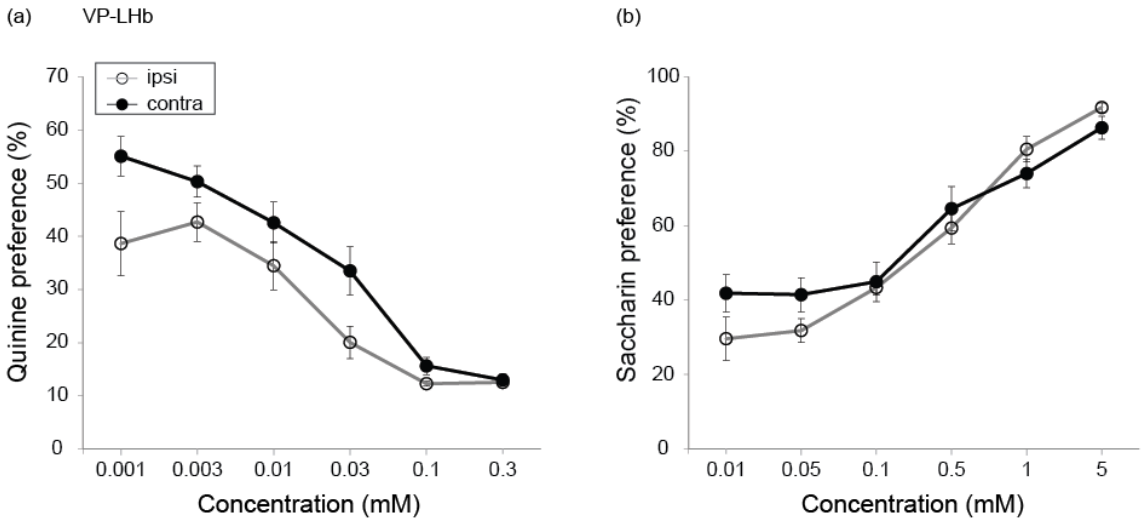

Figure 5.7 Effect of VP-LHb disconnection on taste preference. Lesions of VP-LHb increases (a) quinine preference but does not alter (b) saccharin preference.

Figure 5.8 Lesion placements and CTb labelling. CTb injection sites in the LHb (left side of panels) and lesion placements centred on LHb for the Contra- group in the LH-LHb disconnection study (right side of panels) (B) Lesion placements in LH for contra- and ipsi- groups (left and right side of panels, respectively) for the LH-LHb study. CTb back-labelling of neurons within the LH that project to LHb are represented as brown circles. There were more projection neurons on the same side of the brain as the CTb injection site than the alternate side. These are the neurons that would be 'disconnected' by lesions for the contra- group. (C) Lesion placements in VP for contra- and ipsi- groups (left and right side of panels, respectively) for the VP-LHb study. CTb back-labelling of neurons within the VP and Lateral Preoptic areas (indicated by black outlined areas) that project to LHb are represented as brown circles. Numbers show distance from Bregma. Scale bar represents 1 mm. (D) Photomicrograph of representative CTb injection site in LHb (scale bar: 0.5 mm). (E) Photomicrograph showing Fos (blue arrow) and CTb (red arrow) in LH following vehicle injection (scale bar: 50 µm) (F) Photomicrograph showing Fos (blue arrow) and CTb (red arrows) in LH following yohimbine injection. Note that there is more Fos expression following yohimbine than vehicle injection, and there is no co-localization of Fos and CTb.

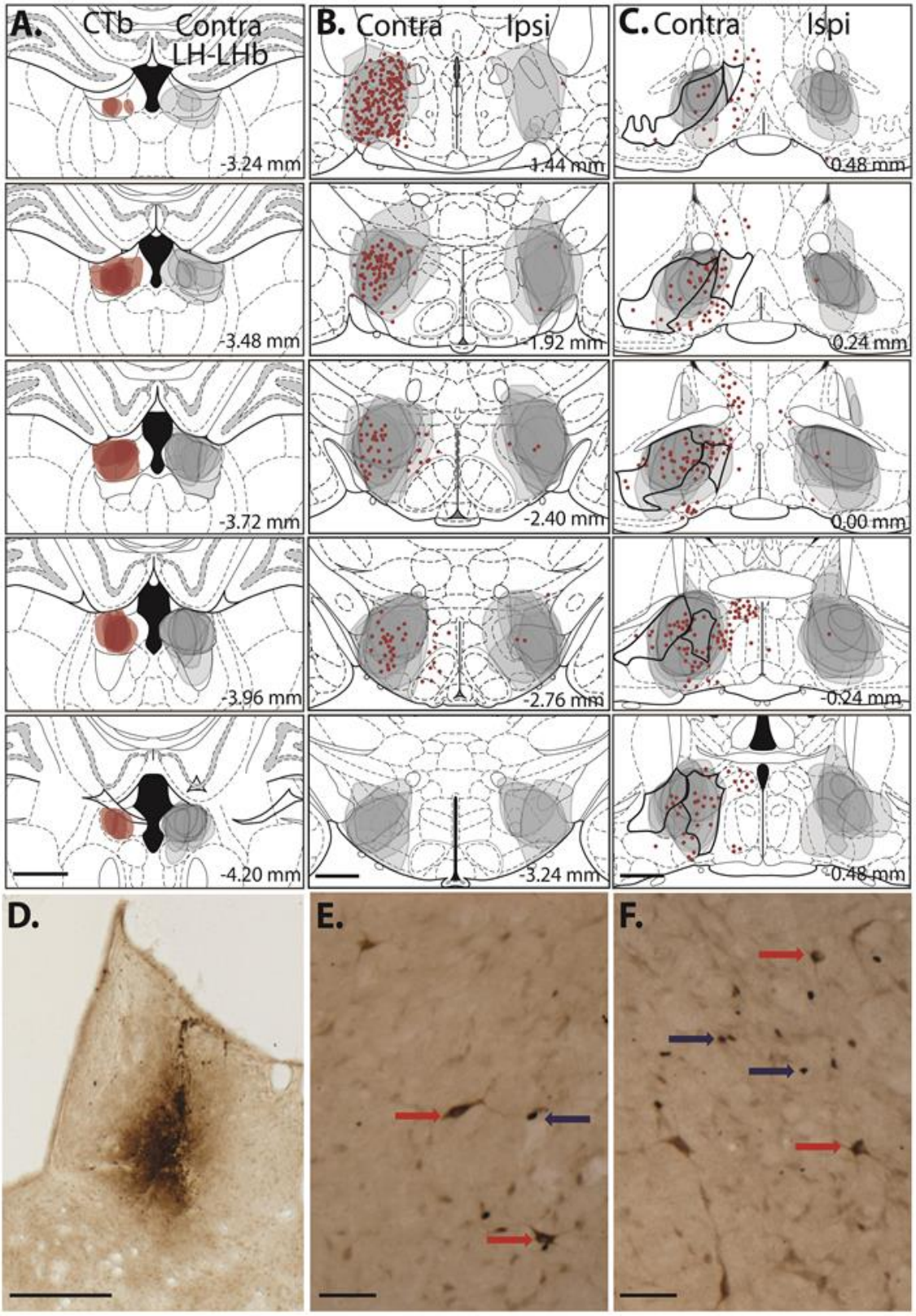
A.
CTb
Contra
LH-LHb
B. Contra
Ipsi
C. Contra
Ispi
-3.24 mm
-1.44 mm
0.48 mm
-3.48 mm
-1.92 mm
0.24 mm
-3.72 mm
-2.40 mm
0.00 mm
-3.96 mm
-2.76 mm
-0.24 mm
-4.20 mm
-3.24 mm
0.48 mm
D.
E.
F.

Table 5.1 Mean ±S.E.M counts of CTb, c-Fos and Fos/CTb neurons per section for control and yohimbine treatments. Asterisks indicate significant difference between treatment groups, $p<0.05$.

	LH			VP	
	control	YOH		control	YOH
CTb	13.5±6.7	9.1 ±4.1		4.8 ±2.3	3.6 ±1.8
c-Fos	7.7±0.8*	25.3±4.3*		2.3 ±0.2	8.3 ±2.6
Fos/CTb	0.1 ±0.1	0.1 ±0.1		0.0 ±0.0	0.1 ±0.1

CHAPTER 6

SUMMARY

Drugs of abuse exert rewarding effects, and it is believed that these rewarding effects mediate drug-taking behavior. Given the importance of the positively reinforcing effects of drugs of abuse, they have been studied extensively from a behavioral, neurochemical, and neuroanatomical standpoint (Koob and Le Moal, 2006). Often neglected is the fact that drugs of abuse, including ethanol, also have aversive effects that occur independently of the drugs' rewarding effects (Verendeev and Riley, 2013). It has been increasingly proposed by several studies that the relative balance between the rewarding and aversive effects of a drug governs the likelihood of drug intake (Riley, 2011). To better understand the neurobiological underpinnings of drug intake and drug addiction, it is important to focus attention on both rewarding and aversive effects and the interplay between these two effects.

I focused my dissertation on one of the most commonly abused drugs, ethanol. The estimated economic cost of excessive drinking was $223.5 billion in 2005 (Bouchery et al., 2011) and ethanol was responsible for 9% of global deaths in 2006 (Shield et al., 2013), highlighting the fact that ethanol exerts a

huge economic and social burden. Although the neural circuitry and neurobiological mechanisms underlying ethanol reward have been well defined (Hyman et al., 2006), the neural circuitry mediating aversion to ethanol is unknown. Preclinical and clinical evidence suggests that ethanol intake is governed by the relative balance between rewarding and aversive properties of ethanol (Verendeev and Riley, 2013). Furthermore, sensitivity to the aversive effects of ethanol has been implicated in limiting voluntary ethanol intake (Green and Grahame, 2008; King et al., 2011; King et al., 2014). Given the prevalence of alcohol use disorders (AUD's), it is important to dissect the neural circuitry that mediates aversive properties of ethanol and that hence can potentially regulate ethanol consumption. Identifying the neural substrates regulating ethanol intake by mediating aversion can help provide new anatomical and pharmacological targets for treating AUD's. In this dissertation, I examined the role of the LHb in aversion to ethanol because of evidence that the LHb encodes aversion by inhibiting midbrain DA neurons (Christoph et al., 1986; Hong et al., 2011). In addition, increasing evidence has implicated the LHb in mediating aversive motivational states (Matsumoto and Hikosaka, 2009a; Stamatakis and Stuber, 2012; Jhou et al., 2013). In Chapter 2 of my dissertation, I show that the LHb plays a crucial role in controlling voluntary ethanol consumption and operant ethanol-seeking, most likely by modulating ethanol-induced aversive conditioning. Further, I show that the role of the LHb in ethanol-induced aversion does not extend to the acute aversive properties of ethanol, but rather is restricted to ethanol-induced aversive learning (Chapter 3). Since my results

indicated that the LHb plays an integral role in ethanol-directed behaviors, I further explored the afferent and efferent structures of the LHb that may mediate the effects of the LHb on ethanol-directed behaviors. In Chapter 4 of my dissertation, I show that the RMTg, which is the major efferent target of the LHb, controls voluntary ethanol consumption by mediating ethanol-induced conditioned aversion. This finding suggests that the LHb and RMTg play tightly coupled roles in regulating ethanol intake. Finally, I investigated the afferents of the LHb that may mediate effects of the LHb on ethanol-associated behaviors (Chapter 5). I found that the LH-LHb projection regulated voluntary ethanol consumption, whereas the VP-LHb projection was not necessary for mediating the role of the LHb on ethanol-directed behaviors. In the following sections, I will describe the implications of these findings and discuss potential avenues for future research.

Role of the LHb in ethanol directed behaviors

The LHb has been implicated in aversive processing and promoting avoidance behaviors (Stamatakis and Stuber, 2012). Interestingly, the role of the LHb in mediating aversion also extends to the aversive motivational state induced by cocaine (Friedman et al., 2010; Jhou et al., 2013). However, whether the LHb is involved in ethanol-induced aversion and intake was previously unknown. I therefore investigated this fundamental question in my dissertation work by studying the effects of the lesions of the LHb on ethanol-directed behaviors. In Chapter 2, I show that lesions of the LHb increase voluntary

ethanol consumption in home cages as well as operant responding for ethanol. The increase in ethanol consumption seen in the LHb-lesioned rats can be attributed to deficits in learning from aversive outcomes of ethanol intake, as is evident in the results of the CTA paradigm. Although ethanol conditioned a robust aversion in both sham- and LHb-lesioned rats, the LHb-lesioned rats extinguished their CTA faster than did the sham-lesioned rats. This finding is consistent with another recent study demonstrating that inactivation of the LHb blocks ethanol-induced conditioned place aversion (Zuo et al., 2015). Further, my results (Chapter 2) show that lesions of the LHb block yohimbine-induced reinstatement of ethanol-seeking, suggesting that the LHb may be necessary for mediating stress-induced relapse to ethanol-seeking. This finding is consistent with a role for LHb in stress-induced behavioral and neurochemical responses (Thornton and Bradbury, 1989; Gill et al., 2013).

Role of the LHb in acute aversive properties of ethanol

The results presented in Chapter 2 demonstrated that the LHb plays a role in ethanol-induced aversion learning. However, the involvement of the LHb in the acute aversive physiologic effects of ethanol was heretofore unknown. Sensitivity to the acute aversive effects of ethanol such as sedation, motor impairment, and ethanol-withdrawal-induced anxiety reduces ethanol intake (Little et al., 1996; White et al., 2002; Doremus-Fitzwater and Spear, 2007). Hence it was important to determine whether increased ethanol intake seen in LHb-lesioned rats could

result from the decreased acute aversive effects of ethanol. In Chapter 3, I found that lesions of LHb did not alter ethanol-induced motor impairment, ethanol-induced sedation, and ethanol-withdrawal-induced anxiety, suggesting that LHb does not play a significant role in mediating the acute aversive effects of ethanol.

An interesting finding of the above results was that LHb lesions increased exploration of open arms, which is indicative of lower anxiety under basal conditions. This observation is in line with previous findings of the effects of LHb inactivation showing a reduction in anxiety under basal as well as heightened stress conditions (Gill et al., 2013). Additional studies are needed to examine whether these lower levels of basal anxiety in LHb-lesioned rats may contribute to increased propensity for ethanol intake. First, a correlation between basal level of anxiety and ethanol-seeking should be established, since extant studies investigating this question have yielded conflicting results (Spanagel et al., 1995; Moller et al., 1997; Henniger et al., 2002). Further, future studies should examine whether the LHb plays a central role in establishing the relation between anxiety and ethanol-seeking. Specifically, this question can be addressed by comparing the relation between basal levels of anxiety and voluntary ethanol intake between normal and LHb-lesioned rats.

Role of the RMTg in ethanol directed behaviors

The LHb negatively modulates midbrain DA and 5-HT systems through a di-synaptic connection involving the RMTg, with the LHb sending glutamatergic

projections to the RMTg, a GABAergic nucleus which then suppresses VTA DA neuron firing (Jhou et al., 2009b; Hong et al., 2011). Like the LHb, the RMTg encodes aversive stimuli; these neurons specifically receive afferent projections from the LHb and project to VTA DA neurons (Jhou et al., 2009a). Also, optogenetic stimulation of the LHb-RMTg projection is negatively reinforcing and produces active, passive, and conditioned avoidance, suggesting that the LHb mediates avoidance responses to aversive stimuli via the RMTg (Stamatakis and Stuber, 2012). Further, the LHb-RMTg pathway mediates aversion to cocaine (Jhou et al., 2013). Thus, it was important to investigate whether the RMTg mediates the effects of LHb lesions on ethanol-related behaviors. In agreement with my hypothesis, I found that RMTg lesions, like LHb lesions, increased voluntary ethanol consumption and accelerated extinction of ethanol-induced CTA, strongly suggesting that the LHb-RMTg pathway may regulate voluntary ethanol consumption by mediating ethanol-induced conditioned aversion (Chapter 4).

Surprisingly, we found that lesions of the RMTg, unlike the lesions of the LHb, did not alter operant ethanol self-administration and yohimbine-induced reinstatement of ethanol-seeking, suggesting that LHb may mediate its effects on these behaviors through direct projections to the VTA or DRN and MnR (Herkenham and Nauta, 1979; Goncalves et al., 2012; Sego et al., 2014). It is interesting that although lesions of the LHb increase both voluntary ethanol consumption and operant ethanol-seeking, the effect of lesion of the RMTg is specifically on voluntary ethanol consumption. Voluntary ethanol consumption in

the home cage when ethanol is freely available examines ethanol “taking” or consummatory behavior, whereas operant ethanol self-administration, in which an animal has to make a response to obtain ethanol, investigates ethanol “seeking” or appetitive behaviors. Neural correlates and circuitry underlying “taking” and “seeking” behaviors are known to be different. For example, a D2 antagonist reduces ethanol-seeking but does not affect ethanol-taking. Conversely, FDA-approved drugs for ethanol dependence, such as acamprostate and naltrexone, decrease ethanol-taking but leave ethanol-seeking unaffected (Brown et al., 1982; Czachowski et al., 2001; Czachowski et al., 2002). These results lend themselves to an attractive hypothesis: the LHb may be a convergence point between “taking” and “seeking” behaviors, since LHb lesions increase ethanol voluntary consumption, as well as operant ethanol self-administration. The RMTg, on the other hand, is only involved in ethanol-taking behavior.

Role of the LHb afferents in ethanol directed behaviors

I investigated in Chapter 5 what afferent input to the LHb was required for mediating the effects of the LHb on ethanol-associated behaviors described in Chapter 2. I found that lesioning the SM which carries the afferents to the LHb increases voluntary ethanol consumption in an IEA paradigm, suggesting that afferents to the LHb underlie its role in generally suppressing ethanol intake. Further, I examined the role of specific afferents to the LHb in behaviors

associated with ethanol intake. Specifically, I looked at the LH-LHb and VP-LHb projections, given the established role of the LH and VP in reward and motivation, including ethanol drinking (Kemppainen et al., 2012; Chen et al., 2013; Chen et al., 2014). I found that disconnecting the LH and LHb increased ethanol-taking but had no effect on ethanol-seeking, yohimbine-induced reinstatement of ethanol-seeking, and ethanol-induced CTA. In contrast, disconnecting the VP and LHb had no effects on any of the tested ethanol-related behaviors. These results suggest that the LH-LHb input may be involved in regulating ethanol-taking.

As discussed above, the neural circuitries underlying taking and seeking behaviors are known to be different. My results suggest that the LH-LHb disconnection selectively influences ethanol-taking behaviors and leaves the ethanol-seeking behavior intact. The afferent circuitry driving the involvement of the LHb in ethanol-seeking behaviors remains to be determined.

Interestingly, LH-LHb disconnection did not alter the acquisition or extinction of ethanol-induced CTA. Although rats with disconnection of the LH-LHb projection seemed to recover a little faster during extinction of ethanol-induced CTA, the differences did not reach statistical significance. This suggests that there may be other mechanisms which contribute to increased ethanol voluntary consumption in LH-LHb cross-lesioned rats. An alternate mechanism could be that the LH-LHb pathway regulates anxiety levels, since the LH and LHb both play important roles in regulating anxiety, although a role for the LH-LHb projection in anxiety-related behaviors is not established (Hakvoort

Schwerdtfeger and Menard, 2008; Gill et al., 2013; Kim et al., 2013). Further, anxiety is an important factor in determining ethanol intake (Spanagel et al., 1995; Henniger et al., 2002). Thus, it is possible that disconnection of the LH-LHb projection alters anxiety levels, which then increases ethanol intake. Additional studies investigating the causal role of the LH-LHb projection in anxiety-related behaviors and ethanol consumption are therefore required. Another possible mechanism is that the LH-LHb disconnection alters the encoding of the rewarding properties of ethanol and hence increases ethanol consumption. In fact, there exists a modest positive correlation between home cage drinking and ethanol-induced conditioned place preference (CPP), an assay typically used to evaluate rewarding effects of drugs, in inbred mice, suggesting that higher ethanol intake may be due to the greater rewarding effects of ethanol (Green and Grahame, 2008). Interestingly, ethanol-induced CPP is notoriously difficult to produce in rats. While there are scattered reports of success after multiple conditioning sessions and prolonged exposure to ethanol (Reid et al., 1985; Bienkowski et al., 1995), there are many more reports of either ethanol-induced aversion (using similar concentrations of ethanol) or a complete failure to induce CPP in rats (Cunningham, 1981; van der Kooy et al., 1983; Asin et al., 1985). There is no consensus approach on how to reliably produce ethanol CPP in rats, and thus there is a specific technical difficulty in evaluating ethanol reward in LH-LHb cross-lesioned rats. One way to overcome this problem is to use a similar LH-LHb disconnection in mice to examine the role of this pathway in ethanol-induced CPP.

Justification of use of permanent lesion techniques

There are well-justified, sound experimental reasons for using permanent, tissue-destroying lesions in these studies. All of the studies in this dissertation require the assessment of two very slowly developing behaviors – escalation of alcohol intake, and extinction of ethanol-induced CTA. It takes many days for control rats to increase voluntary ethanol intake, and the effects of lesion on this escalation are apparent only after a number of days. Such is also true for extinction of CTA, which develops slowly over a number of days. Precisely for this reason, I think that permanent lesions, rather than transient inactivation offered by optogenetic/chemogenetic techniques, are a better choice for these experiments. Techniques effecting a permanent change of the neural circuit, rather than a transient and reversible one, are more likely to reveal behavioral differences that emerge in an incremental, cumulative fashion. Further, optogenetic/chemogenetic techniques suffer from the risk of partial inactivation. Also, the LHb, which is an elongated structure, extends 1mm anterior-posteriorly. Thus, permanent lesions are better for inactivating the entire anterior-posterior extent of the LHb. However, permanent lesions suffer from disadvantages including long-term compensation, which occurs over time.

Translational relevance

Identifying the neural circuitry underlying aversion to ethanol can be important for providing targets in limiting excessive ethanol intake in alcohol use

disorders (AUDs). DBS of the LHb reduces cocaine seeking in rats (Friedman et al., 2010). Given our results, I would hypothesize that DBS of LHb will also reduce ethanol-seeking. DBS of the afferent bundle of LHb, SM, has been used in a patient with treatment-resistant depression, providing a preliminary safety and efficacy validation of this approach (Sartorius et al., 2010). In fact there is a clinical trial taking place at the Icahn School of Medicine at Mount Sinai investigating the efficacy of DBS of the LHb in treatment-resistant depression. In light of the findings obtained in this dissertation, the LH and RMTg should also be investigated as potential DBS targets for reducing excessive ethanol-taking.

Future directions

Recording from the LHb during ethanol-induced CTA

An important direction of future research is to investigate the neural encoding in the LHb before and after ethanol-induced CTA. Currently, these experiments are underway in the laboratory. The results show that the baseline firing rate of LHb neurons is elevated after ethanol-induced CTA. Also, event-related (cue-evoked & lever press-evoked) LHb firing is increased. The event-related increase in LHb firing has a strong correlation with behavioral response latencies, suggesting that the LHb neural activity may encode and drive ethanol-induced CTA (Tandon et al., Manuscript submitted).

Functional role of LHb-raphe projections

The LHb sends direct projections to the midbrain 5-HT centers, including the DRN and MnR (Herkenham and Nauta, 1979; Sego et al., 2014). However, the functional role of this direct projection with regard to drug intake has not been explored. 5-HT is crucial for regulation of mood and emotions, with reduced levels of 5-HT leading to a negative mood (Coccaro et al., 1990). Negative mood and stress exposure have both been linked to high rates of relapse after periods of drug abstinence (McKay, 2011). Two lines of evidence suggest that the LHb-raphe projections may play a role in stress-induced relapse of drugs. First, electrical stimulation of LHb inhibits firing of 5-HT neurons, suggesting a negative modulation of 5-HT transmission in the brain (Wang and Aghajanian, 1977). Second, aversive stimuli such as stressors activate the LHb, and activation of the LHb has been associated with drug relapse (Wirtshafter et al., 1994; Timofeeva and Richard, 2001; Matsumoto and Hikosaka, 2007; Brown and Shepard, 2013). Thus, it is plausible that stress-induced activation of LHb inhibits downstream 5-HT neurons, producing a negative mood state which triggers drug-seeking. Further, there is increasing evidence that LHb-raphe projections may convey value state information (Proulx et al., 2014). For example, in zebrafish, the LHb–MnR pathway is necessary for avoidance learning, suggesting the importance of this projection in learning aversively motivated behaviors (Amo et al., 2014). Thus, it is imperative to examine whether the LHb to DRN and MnR projections are involved in drug-related behaviors, given the role of this projection in stress-induced behavioral responses and encoding negative value states.

Role of the LHb in ethanol dependence and withdrawal

A future direction of particular relevance to this current work is to examine the role of the LHb in ethanol dependence and withdrawal. Although I have shown that lesions of the LHb increase ethanol intake during IEA, it is important to note that this paradigm does not induce ethanol dependence in rats, but rather models transition from social to excessive drinking (Carnicella et al., 2014).

Models of ethanol dependence include ethanol vapor exposure and ethanol liquid diet (Becker, 2008). Studies using *in vivo* microdialysis and fast scan cyclic voltammetry have shown that withdrawal from these paradigms is associated with reduced extracellular DA levels in the terminal fields (Weiss et al., 1996; Karkhanis et al., 2015; Rose et al., 2015). According to the opponent process model of drug-seeking, the withdrawal-induced DA hypofunction is dysphoric/aversive and promotes further drug-seeking to restore DA levels to normal (Solomon and Corbit, 1974; Weiss et al., 1996). However, the mechanisms causing the hypodopaminergic state during withdrawal are not well understood.

The hypothesis that should be tested is that ethanol withdrawal causes overactivity of the LHb, which increases RMTg-induced inhibition of VTA DA neurons. The increased activity of the LHb may underlie the DA hypofunction and thus contribute to withdrawal-induced excessive ethanol-seeking. Future studies should investigate LHb and RMTg neuronal firing during ethanol withdrawal and also examine whether pharmacologic/optogenetic manipulations of the LHb and RMTg alter increased ethanol-seeking during withdrawal.

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